Title: Development and Validation of Ultra-high-performance Liquid Chromatography-tandem Mass Spectrometry Methods for the Simultaneous Determination of Beauvericin, Enniatins (A, A1, B, B1) and Cereulide in Maize, Wheat, Pasta and Rice

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Development and validation of ultra-performance liquid chromatography tandem mass-spectrometry methods for the simultaneous determination of beauvericin, enniatins (A, A1, B, B1) and cereulide in maize, wheat, pasta and rice

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Abstract

Rapid and accurate UPLC-MS/MS methods for the simultaneous determination of beauvericin and the related enniatins (A, A1, B, B1), together with cereulide were successfully developed and validated in cereal and cereal-based food matrices such as wheat, maize, rice and pasta. Although these emerging foodborne toxins are of different microbial origin, the similar structural, toxicological and food safety features provided rationale for their concurrent detection in relevant food matrices. A Waters Acquity UPLC system coupled to a Waters Quattro Premier XE™ Mass Spectrometer operating in ESI+ mode was employed. Sample pretreatment involved a fast and simple liquid extraction of the target toxins without any further clean-up step. For all toxins the sample preparation resulted in acceptable extraction recoveries with values of 85-105% for wheat, 87-106% for maize, 84-106% for rice and 85-105% for pasta. The efficient extraction protocol, together with a fast chromatographic separation of 7 min allowed substantial saving costs and time showing its robustness and performance. The validation of the developed method was performed based on Commission Decision 2002/657/EC. The obtained limits of detection ranged from 0.1 to 1.0 $\mu g.kg^{-1}$ and the limits of quantification from 0.3 to 2.9 $\mu g.kg^{-1}$ for the targeted toxins in the selected matrices. The obtained sensitivities allow detection of relevant toxicological concentrations. All relative standard deviations for repeatability (intra-day) and intermediate precision (inter-day) were lower than 20%. Trueness, expressed as the apparent recovery varied from 80 to 107 %. The highly sensitive and repeatable validated method was applied to 57 naturally contaminated samples allowing detection of sub-clinical doses of the toxins.

Keywords: Cyclic depsipeptides; Emerging mycotoxins; *Fusarium*; Cereulide; LC-MS analysis; Solvent extraction.

1 Introduction

Contamination of food and feed with toxins is one of the main concerns in the food industry. Both bacteria and fungi are capable of producing microbial metabolites in food and feed under the appropriate environmental conditions. These toxins can enter the food chain directly through contaminated food or indirect through the presence of contaminants in food of animal origin derived from animals, which were fed with contaminated grains. Even though several pre-and post-harvest efforts such as sorting, kernel and hand sorting are made in order to prevent and control bacteria and fungi, the produced toxins can remain active even after very harsh treatments [1]. In addition, the toxins are stable under the most common conditions used in food processing and can consequently be found in the prepared products [2, 3]. Contamination with toxins of fungal and bacterial origin may lead to acute poisoning or have long-term negative consequences on the health of both human and animals [4]. Besides the health risk, contaminated food and feed causes financial losses with enormous economic impact all over the world. Therefore, an assessment of the presence and impact of these harmful toxins is imperative and starts with developing methods for their detection and quantification.

Mycotoxins are toxic secondary metabolites produced by several fungi, mainly *Aspergillus spp*., *Penicillium spp*. and *Fusarium spp*. [5]. Acute effects (short-term) as well as chronic effects (long-term) have been reported after exposure to these toxic fungal metabolites. Mycotoxins are common contaminants of many grains like wheat, barley, maize, and rice. The most prevalent mycotoxins such as zearalenone, aflatoxins, ochratoxin A, trichothecenes, deoxynivalenol have been frequently studied. Unfortunately, there is limited data on the toxicity and occurrence of the so-called 'emerging' mycotoxins. These mycotoxins are neither routinely determined, nor legislatively regulated. Examples are beauvericin (BEA) and the related enniatins A, A1, B, B1 (ENNs), both produced by several *Fusarium* species. Their presence has been reported in cereals from several countries and in human biological fluids [6-8]. Recently EFSA published an opinion on the presence of ENNs and BEA in food and feed, but the lack of relevant toxicity data prevented a risk assessment [9].

In addition to mycotoxins, bacterial toxins are of global concern, mainly related to foodborne illnesses. The latest report of EFSA on zoonoses, zoonotic agents and foodborne outbreaks revealed that bacterial toxins encounter for 16.1 % of all reported foodborne outbreaks caused by microbial contamination. This figure shows an increase of 60% over a period of 5 years [10]. Foodborne bacterial pathogens that

are well known as toxin producers are *Staphylococcus aureus*, *Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus*. Of multiple toxins produced by these pathogens the most resistant is the emetic toxin cereulide. *Bacillus cereus* is a gram-positive spore-forming pathogen that causes two types of food poisoning syndromes: an emetic (vomiting) intoxication and a diarrheal infection. The emetic syndrome, which is inducted by the toxin cereulide results in vomiting a few hours after ingestion of the contaminated food [11]. Although *B. cereus* can be present in various food products, most reported food poisoning cases were associated with rice and pasta dishes. This emetic toxin is often related to acute food poisoning, occasionally even with a fatal outcome [12, 13]. Cereulide is characterized by its resistance to extreme pH and heat conditions, and resistance to digestion enzymes like pepsin and trypsin [2]. Consequently, it survives food processing and preparation and retains activity during gastrointestinal passage [2, 14]. This illustrates the high importance of a rapid identification and detection of the emetic toxin.

BEA, ENNs and CER are all cyclic depsipeptides with ionophoric properties. Their apolar nature gives them the ability to incorporate into lipid bilayers of cell membranes. Hereby they create cation selective channels that increase the permeability for cations, resulting in disturbances of the physiological cation level in the cell [15, 16]. CER is a cyclic dodecadepsipeptide (twelve-membered) while BEA (and ENNs) are smaller cyclic hexadepsipeptides (six-membered) [17, 18]. The chemical structures of beauvericin and enniatins and cereulide are depicted in Figure 1. Both the bacterial toxin CER and the fungal toxin BEA (and the related ENNs) are regarded as emerging health hazards and their striking similarities should allow a common approach towards the development of a detection technique. The possible co-occurrence of the different toxic compounds in one matrix implies a potential risk for additive, synergic or antagonist toxic effects. Considering the risks to human and animal health, the determination of the occurrence of these medium-sized cyclic depsipeptides in food and feed is imperative. Their potential presence at low levels is of special relevance to food safety [19, 20].

The risk associated with the presence of these toxins initiated the search for more sensitive analytical methods applicable in various matrices. Santini et al. published a review that summarizes techniques used for extraction and quantification of beauvericin and fusaproliferin in food matrices [21]. It became clear that in the search for low detection levels, mass spectrometry has been increasingly used to achieve this goal. The commonly exerted steps regarding the sample preparation are extraction with solvents sometimes followed by an extra clean-up with different types of columns and/or a filtration step. Over the past few year, several methods have been developed for BEA and/or ENNs using mainly

acetonitrile, chloroform, methanol or a mixture with water as extraction solvent [22-26]. Alternately, Ambrosino et al. optimized a sample preparation involving supercritical fluid extraction (SFE) with supercritical CO_2. SFE with methanol as modifier provided similar extraction yields compared to conventional extraction protocols [27]. Although this procedure required less organic solvent, it has not been used regularly. Most papers focused on the detection in cereal (based) samples, but few papers reported method development for biological samples like hen eggs and pig plasma [24, 28, 29]. Sample preparation time and detection levels significantly improved from 1-50 $mg.kg^{-1}$ to trace analysis at low $\mu g.kg^{-1}$ levels by switching from HPLC with UV or DAD detection to UPLC with (tandem) MS detection [26, 30]. Concerning CER, the use of LC-MS is preferred over the HEp-2 cell assay and the boar sperm motility bioassay. Parallel to BEA and ENNs, improved sample preparation is essential for an accurate quantification. Methods developed for determination of cereulide revealed similar sample preparation involving extraction solvent followed by a filtering and/or centrifuging step. Among the increasing number of studies focusing on the determination of the emerging *Fusarium* mycotoxins, none of the papers included cereulide as target compound. Nevertheless, these toxins have been reported in similar kinds of food matrices, more specifically cereals and cereal-based food products.

The goal was to develop and validate simple sample preparations with a minimum of additional clean up steps for the simultaneous analysis with LC-MS/MS. The selection of the matrices was based on relevance of the matrix with respect to (myco)toxin contamination. Since food poisoning caused by CER is often associated with rice and pasta dishes, these matrices were included. Concerning BEA and ENNs, mainly grains such as wheat and maize are reported and therefore added. The selected matrices are relevant sources of contamination, which might give insight into co-occurrence of CER and BEA and the related ENNs. Such approach will foster efforts of studies of mixture toxicities, which is one of the primary targets in current regulatory toxicology.

2 Materials and methods

2.1 Reagents and chemicals

Methanol (absolute, LC-MS grade), acetonitrile (HPLC grade and LC-MS grade) and glacial formic acid (99%, ULC-MS) were purchased from BioSolve BV (Valkenswaard, the Netherlands). Methanol (HiPerSolv Chromanorm HPLC grade) was obtained from VWR International (Zaventem, Belgium). Ammonium acetate was supplied by Merck (Darmstadt, Germany). Water was purified on a Milli-Q® SP Reagent water system from Millipore Corp (Brussels, Belgium). Ultrafree®-MC centrifugal filter devices (0.22 µm) were obtained from Millipore (Bredford, MA, USA).

2.2 Standard solutions

BEA, ENN A, ENN A1, ENN B, ENN B1 (1 mg, solid standard) and valinomycin (VAL) (10 mg, solid standard) were purchased from Sigma-Aldrich (Diegem, Belgium), while CER (1 mg, solid standard) was supplied by Chiralix (Nijmegen, The Netherlands). Primary stock solutions were prepared by dissolving the solid standard in acetonitrile (1 $mg.ml^{-1}$). All stock solutions were stored at −20°C, except VAL was stored at 4 °C. Working solutions of 10 $\mu g.mL^{-1}$ were prepared in acetonitrile, stored at 4 °C and renewed monthly. Mixture solutions (BEA, ENNs and CER) were prepared prior to each experiment by diluting the working solution in acetonitrile.

2.3 Naturally contaminated samples

A total of 57 food and feed samples were randomly collected in Belgium. Rice (n = 12) and pasta (n = 12) samples were collected from Belgian supermarkets in 2015. Wheat (n = 10) and maize (n = 23) samples were randomly collected from several European and African countries such as Nigeria, Zimbabwe, Poland and Hungary. The samples were collected after harvest and immediately stored at room temperature until analysis. The samples were quantified with matrix-matched calibration curves using blank samples. The unknown samples as well as the spiked samples of the calibration curve were treated as described below (2.5).

2.4 LC-MS/MS

LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Waters Quattro Premier XE^{TM} Mass Spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface (ESI). For data acquisition and processing, Masslynx and Quanlynx software 4.0 (Waters) were used. Chromatographic separation was achieved on an Acquity UPLC BEH C_{18} column (1.7 µm, 2.1 mm x 50 mm) with a flow rate of 0.3 mL.min^{-1}. The column and auto sampler temperature were set at 30 °C and 20 °C, respectively. A mixture of ACN and MeOH (80/20, v/v) was used as organic solvent in the mobile phase. Mobile phase consisted of eluent A (water/organic solvent, 95:5, v/v) and eluent B (organic solvent/water, 98:2, v/v) both containing 1 mM ammonium acetate and 0.3 % formic acid. Gradient elution allowed separation in 7 min. The gradient elution program initiated with 70 % eluent B which was linearly increased to 100 % in 3 min. From 3 to 5 min an isocratic phase of 100 % eluent B was maintained. In 0.1 min the gradient switched again to 70 % eluent B and was maintained for 2 min to equilibrate the column. The MS analyses were carried out using multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI^{+}). In order to optimize the MS parameters, tuning solutions of each compound (10 ng.µL^{-1}) were directly infused (flow rate of 10 µL.min^{-1}) into the mass spectrometer. The two most abundant product ions were selected. Ideal fragmentation conditions were accomplished by varying the cone voltage and collision energies for each compound and can be found in Table 1. The product ion with the highest intensity and S/N ratio was selected for validation and quantification, whereas the second production ion was used for confirmation. The antibiotic valinomycin (VAL) structurally resembles CER and served therefore as internal standard [18, 31].

2.5 Sample preparation and extraction

Initially, the food and grain samples were homogenized and ground using a M20-grinder (Ika Werke, Staufen, Germany). Then, 2.000 g ± 0.005 g portions of the homogenized samples were transferred into 50 mL extraction tubes. Each sample was fortified (spiked) with a fixed concentration (10 µg.kg^{-1}) of VAL internal standard and mixed with a vortex for 0.5 min. After leaving the samples 30 min for equilibration, 10 mL of extraction solvent was added. Samples were extracted for 20 min using an overhead shaker (Agitelec, J. Toulemonde and Cie, Paris, France) and subsequently centrifuged for 10 min at 4000 x g. An aliquot of 8 mL supernatant was transferred and evaporated to dryness under a gentle stream of nitrogen using a Turbovap LV Evaporator (Biotage, Charlotte, USA). After solvent evaporation, the extract was reconstituted with 300 µL of the injection solvent (eluent A/eluent B,

20:80, v/v), vigorously vortexed for 1 min and filtered through an Ultrafree-MC centrifugal device (Millipore, Bedford, MA, USA) prior to injection in the LC-MS/MS system.

2.6 Method validation

2.6.1 Validation design

For validation study, Commission Decision 2002/657/EC, Commission Regulation 401/2006/EC and ICH guidelines were used as guidance. Since no reference material was available, spiked blank samples of the corresponding matrix were used for validation of the multi-method for wheat, maize, rice and pasta. During method validation the performance characteristics of the method were evaluated by a set of parameters: linearity, apparent recovery (R^{app}), repeatability (intra-day RSD_r), intermediate precision (inter-day RSD_R) and measurement uncertainty [32]. Determination of limit of detection (LOD) and limit of quantification (LOQ) was based on ICH guidelines [33]. All validation parameters were calculated using the response (ratio of peak area of analyte to peak area of internal standard valinomycin). Calibration curves were obtained by plotting the response of each analyte against the spiked concentration levels. For confirmatory methods, 4 identification points should simultaneously be fulfilled to assure appropriate certainty in identification: 1 precursor and at least 2 products ions should be monitored, both with a signal-to-noise (S/N) ratio more than 3, the relative intensities of the detected ions should correspond with those of the calibration within accepted deviations and the relative retention time (with regard to the internal standard) of the detected ions must range within a margin of 2.5% [32].

2.6.2 LOD, LOQ and linearity

Limit of detection (LOD) and limit of quantification (LOQ) were experimentally determined according to the ICH guidelines [33]. Therefore, blank samples were spiked with decreasing concentrations of the toxins of interest and treated as described in 2.5. For this purpose, the selected concentration range was close to the expected LOD and LOQ levels determined during method optimization. This experiment was conducted in three independent replicates for each matrix. Subsequently, a calibration curve was constructed and LOD and LOQ were calculated by respectively 3.3 times and 10 times the standard deviation of the response divided by the slope of the calibration curve. In addition, the peak shape and the S/N ratio (at least 3 for LOD and 10 for method LOQ) were evaluated for calculated LOD and LOQ.

Since the linear range of most analytical instruments is known to be limited, the linearity should be assessed. The calibration curve starts around the calculated LOQ and covers a concentration range based on experimental data obtained during method development as no legal limits exist for CER, BEA and ENNs. The linearity of the calibration curves was expressed using the coefficient of determination (R^2) and confirmed by means of the lack-of-fit test (SPSS) [34].

2.6.3 Accuracy and measurement uncertainty

For accuracy and measurement uncertainty blank samples of each matrix were spiked in triplicate on low, medium and high concentration levels with the different toxins. This procedure was executed on 3 consecutive days. Accuracy is studied as two components: trueness and precision. Trueness can be expressed as bias (%) or as apparent recovery (%). Since no certified reference material was available, the apparent recovery (R^{app}) was assessed by addition of known amounts of the analytes to a blank matrix. The apparent recovery (%) is defined as the ratio of the observed concentration for the spiked sample, calculated from the matrix-matched calibration curves, divided by the reference or spiked concentration. For precision, repeatability (intra-day precision) and intermediate precision (inter-day precision) were evaluated by calculating the relative standard deviation (RSD), respectively RSD_r and RSD_R using one-way ANOVA. To report analytical results with respect to their measurement uncertainty, 3 concentration levels (low, medium, high) were determined and the measurement uncertainty was estimated at that level. This uncertainty is the range within the analytical result is likely to fall and depends on the inherent "trueness" and precision of the analytical method. The combined standard uncertainty (u_c) is equal to the positive square root of the intermediate precision (RSD_R) and the bias of the analytical method, which is associated with the uncertainty of the purity of the standards ($U[C_{ref}]$), the accuracy of the bias (S_{bias}) and the root mean square of the bias (RMS_{bias}). Measurement uncertainty was expressed as *U*, the combined expanded measurement uncertainty, using a coverage factor k = 2, providing a level of confidence of approximately 95 %.

$$U = 2 \cdot u_c = 2 \cdot \sqrt{RSD_R^2 + U[C_{ref}]^2 + S_{bias}^2 + RMS_{bias}^2}$$

2.7 Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2010, IBM SPSS Statistics 22 and GraphPad Prism 6.

3 Results and discussion

3.1 Optimisation of LC-MS/MS parameters

The method development was initiated by optimization of MS/MS parameters by introducing a constant flow (10 µL.min^{-1}) of the individual analyte (10 ng.µL^{-1}) into the ion source using a syringe infusion pump. Ideal fragmentation conditions were accomplished by varying the cone voltage and collision energies (Table 1).. Promoting the formation of $[M+NH_4]^+$ adducts led to higher signal intensities, hence ammonium adducts were chosen as precursor ions. Initially, the three most abundant product ions (including the $[M+H]^+$ ion) for each compound were selected. After optimization of the sample preparation, the two most intense transitions were further used for quantitative and qualitative purposes. Three columns, namely Acquity UPLC BEH C_{18} (1.7 µm, 2.1 mm X 50 mm), Acquity UPLC BEH C_{18} (1.7 µm, 2.1 mm X 100 mm) and Symmetry C_{18} (5 µm, 2.1 mm x 150 mm) were tested for chromatographic parameters, such as peak shape and resolution. The Acquity UPLC BEH C_{18} column (50 mm) as stationary phase provided a good separation and shortened the analysis time. Furthermore, various mixtures of solvents such as methanol, acetonitrile, and methanol-acetonitrile as mobile phase were tested. Based on peak intensity, shape and resolution, a mixture of ACN and MeOH (80/20, v/v) was used as organic solvent. Preliminary experiments indicated that the use of ammonium acetate and formic acid improved the efficiency of the MS ionization of the toxins. Finally, the optimized mobile phase consisted of eluent A (water/organic solvent, 95:5, v/v) and eluent B (organic solvent/water, 98:2, v/v) both containing 1 mM ammonium acetate and 0.3 % formic acid. To further increase sensitivity, different column temperatures (25 °C - 40 °C) and flow rates (0.2 mL.min^{-1} and 0.3 mL.min^{-1}) were tested. A gradient eluent program at a flow rate of 0.3 mL.min^{-1} and a column temperature of 30 °C resulted in a better separation and peak symmetry. Total ion chromatograms of the analytes of a spiked rice sample at 100 µg.kg^{-1} are shown in Figure 2.

3.2 Optimisation of the sample preparation

During the optimization of the extraction procedure, the performance of the extraction was evaluated by extraction yield experiments. Therefore, blank samples were spiked in triplicate at one concentration level before and after extraction. Calculations were performed by comparing mean peak areas of the toxin in samples spiked before and after extraction. Based on literature and overall physicochemical properties of the target toxins different proportions of acetonitrile/water and methanol/water were investigated in order to achieve acceptable extraction recoveries [35-38]. In this study the best

compromise for the simultaneous extraction, based on extraction recovery was achieved by using 100 % MeOH for rice, while for the other matrices ACN/H_20 (84/16, v/v) gave the best recovery results. For further clean-up, the use of SPE cartridges (Oasis HLB^{TM}), membrane filters (Filter Paper Circles MN 617 11 cm diameter, Whatman^{TM} glass microfiber filters circles 21 mm diameter), centrifugal filter devices (Millipore Ultrafree®-MC centrifugal filter devices 0.22 µm) and an n-hexane defatting step was investigated. The performance of the additional clean-up step was again evaluated by extraction recovery experiments. Only the use of centrifugal filters prior to LC-MS/MS analysis obtained cleaner sample extracts with a comparable recovery (results not shown). Clean-up procedures using n-hexane, membrane filters and SPE resulted in lower or comparable recoveries. Since a simple liquid extraction is less time-consuming and allows reaching similar recovery results, the clean-up steps with n-hexane and SPE were omitted. Recovery data for the different matrices and the different toxins extracted with the selected solvents can be found in Figure 3. The recoveries of all toxins from the four tested matrices, were close to 100% (ranging between 84% and 106%), with low SD values.

3.3 Method validation

3.3.1 LOD, LOQ and linearity

For each matrix, calibration curves were constructed in triplicate by spiking blank samples with increasing concentrations around the expected LOD. Based on these calibration curves the LOD and LOQ values were calculated. Consequently, the mean recoveries and the associated repeatability was verified for the calculated LOQ. Only LOQ values with mean recoveries within the range 70–110% and an associated repeatability $RSD_r \leq 20\%$ were accepted [32]. The LODs ranged from 0.1 to 1.0 µg.kg^{-1} and the LOQs from 0.3 to 2.9 µg.kg^{-1}.

Based on preliminary experiments during method development and data found in literature, concentration ranges were selected for the different toxins and the different matrices. The level of linearity of the calibration curve is crucial for the quality of your method. Therefore an appropriate regression model should be selected, preferably a linear regression model [34]. According to the coefficients of determination (R^2), with the lowest observed value being 0.978 for ENN B in wheat, calibration curves revealed good linearity within the selected range for all analytes. Furthermore, a lack-of-fit test was carried out to asses if the regression model fits the data. *p*-values above 0.05 demonstrated no lack of fit of the linear model within the selected range. These results ascertains the linearity for the compound within the selected ranges [34]. In addition to the lack-of-fit test an evaluation of the residual plot was done. If individual residuals deviate by more than ±20% from the

calibration curve, weighted linear regression ($1/x^2$) was used [39]. An overview of the linearity data of the matrix-matched calibration curves is shown in Table 2. By lowering the highest concentration of ENN A in wheat, maize and rice from 400 to 200 $\mu g.kg^{-1}$, the linearity improved remarkably especially when preforming a lack-of-fit test. For pasta, the concentration ranges are smaller compared to the other matrices. This adjustment increased both trueness and linearity while still covering the relevant concentration range for dry pasta samples.

3.3.2 Accuracy and measurement uncertainty

The trueness was evaluated by recovery experiments and results were reported as apparent recovery (%). Note the difference between the terms 'recovery' and 'apparent recovery'. Recovery is related to the yield of the extraction stage and therefore named extraction recovery in this paper, whereas apparent recovery is used to denote ratio of the observed value for the spiked sample, obtained via a calibration graph, divided by the reference value [40]. Hence, blank samples were spiked with increasing concentration of the toxin standards prior to extraction and analyzed by the method described above. All values varied from 80 to 107 % and are thus in good agreement with the guideline ranges (80–110%) of 2002/657/EC [32]. Results are summarized in Table 3.

Validation of analytical methods for quantitative determination includes an investigation of precision. Precision was considered at two levels: repeatability (intra-day) and intermediate precision (inter-day). The reported RSD_r-values for repeatability are based on 3 determinations for low, medium and high concentration levels within one day. To expresses variations between different days (intermediate precision) the procedure was repeated on three days. Repeatability (RSD_r) and intermediate precision (RSD_R) ranged from 1.7% to 20% and 2.8% to 20%, respectively. Consequently, the Horwitz equation ($RSD_r = 2/3(2^{[1-0.5 \log C]})$; $RSD_R = 2^{[1-0.5 \log C]}$, where C is the concentration, expressed as a mass fraction) was used to evaluate these RSD values. As described in commission decision 2002/657/EC, the Horwitz Equation gives unacceptable high values for concentrations lower than 100 $\mu g.kg^{-1}$. Therefore, the RSD for concentrations lower than 100 $\mu g.kg^{-1}$ shall be as low as possible [32]. Overall, the RSD values never exceeded the level calculated by the Horwitz equation and thus the method appears to be both repeatable and accurate for all matrices.

Next, the expanded uncertainty *U*, expressed as percentage (*U* %) was calculated to express the

uncertainty of the measured results. *U* was determined for each toxin on three concentration levels. If no certificate of analysis of the reference standards is available, an arbitrary level of $\sqrt{3}$ is chosen for the uncertainty related to the reference standard $U[C_{ref}]$. This high $U[C_{ref}]$ term in the calculation leads to higher values for *U*. Additionally, a high intermediate precision resulted in high *U* values. All *U* values ranged from 5.6 % to 49 % (Table 3). In general, the highest values for *U* were found for maize.

Judging from the results of this detailed validation, the procedures are suitable for the simultaneous determination of the target toxins. The sample preparation was minimized to a simple one-step liquid extraction, which enables the preparation of a high number of samples in a relatively short time. The similar structure and behaviour of the target toxins, avoided loss of sensitivity that often comes with multi analyte methods. All molecules undergoing ionization in the positive ion mode, formed abundant $[M + NH4]^+$ adducts when adding ammonium acetate to the mobile phase. As the modifiers (ammonium acetate and formic acid) influences the target molecules in the same positive way, no compromises had to be made. Similarly, the total analysis time could be reduced due to a short toxin extraction and an efficient LC separation which contributes to the potential to rapidly screens samples. The results show that the LC-MS/MS method is very efficient, sensitive and rapid for the quantification of the target toxins and furthermore, the methodology enabled detection at low detection limits without the need for additional clean-up. As proof of principle, 57 samples were tested.

3.4 Analysis of naturally contaminated samples

The suitability of the optimized and validated methods was finally tested by analyzing 57 naturally contaminated samples The samples were quantified against matrix matched standards. The results are reported in the form 'x ± U' where 'x' is the best estimate of the

true value of the concentration (the analytical result) and 'U' is the expanded uncertainty. Results from the occurrence of CER, BEA and ENNs in the analyzed samples are represented in Table 4. No CER was detected in any of the samples which could be expected since the occurrence of cereulide is usually related to cooked dishes or leftovers [12, 41]. Generally, the level of contamination was low especially for BEA, ENN A and ENN A1, except for maize where in 74 % of the samples BEA was detected up to 209.0 ± 39.7 $\mu g.kg^{-1}$. No ENN A was detected in the samples and only traces of ENN A1 (<14.6 ± 3.9 $\mu g.kg^{-1}$) were found in maize and wheat. No toxins were detected in the rice samples. ENN B and ENN B1 were the mycotoxins most found and levels ranged from 2.8 ± 1.3 to 195.5 ± 47.0 $\mu g.kg^{-1}$ and 1.9 ± 0.7 to

42.5 ± 15.4 µg.kg^{-1}, respectively. Samples containing ENN B were generally also contaminated with ENN B1. The highest levels for each individual toxin recorded were 209.0 ± 39.7 µg.kg^{-1} (BEA), 14.6 ± 3.9 µg.kg^{-1} ENN A1, 195.5 ± 47.0 µg.kg^{-1} (ENN B) and 42.5 ± 15.4 µg.kg^{-1} (ENN B1). The methods were considered to be suitable for use since the measured concentrations are within the validated linear concentration ranges. Although no general conclusions can be drawn concerning the occurrence, the preliminary data of 57 samples tested in the current study suggested that ENN B, B1 and BEA are more abundant contaminants than ENN A and A1 in the selected matrices. These results suggested that in the future our method could be employed in the screening of BEA, ENNs (A, A1, B, B1) and CER in cereals and cereal-based products.

4 Conclusion

Quantitative LC-MS/MS methods applicable for the simultaneous determination of CER, BEA and ENNs in maize, wheat, pasta and rice have been developed. Extensive validation of the method was done for the target toxins in different matrices. Good values for extraction recovery (higher than 80 %) and precision (RSD less than 20.1%) were obtained. The major strengths of the proposed methods are being rapid and simple for all target toxins. Finally, 57 commercially available cereal-based foodstuffs were analyzed with the developed method proving suitability for the intended use. No cereulide was detected in the analysed samples, which is not surprising as cereulide is more likely to occur in leftovers of rice and pasta dishes upon active growth of *B. cereus* during improper holding and storage. 33% (19/57) of the samples were contaminated with ENN B. In 58% (33/57) of the samples at least one of the mycotoxins was detected. None of the commercially available rice samples were contaminated with the target toxins. Since it is likely that more than one toxin is present, a multi-toxin analysis suitable for various matrices helps to monitor the contamination risk. In the future these methods can provide information on the occurrence of these toxic metabolites.

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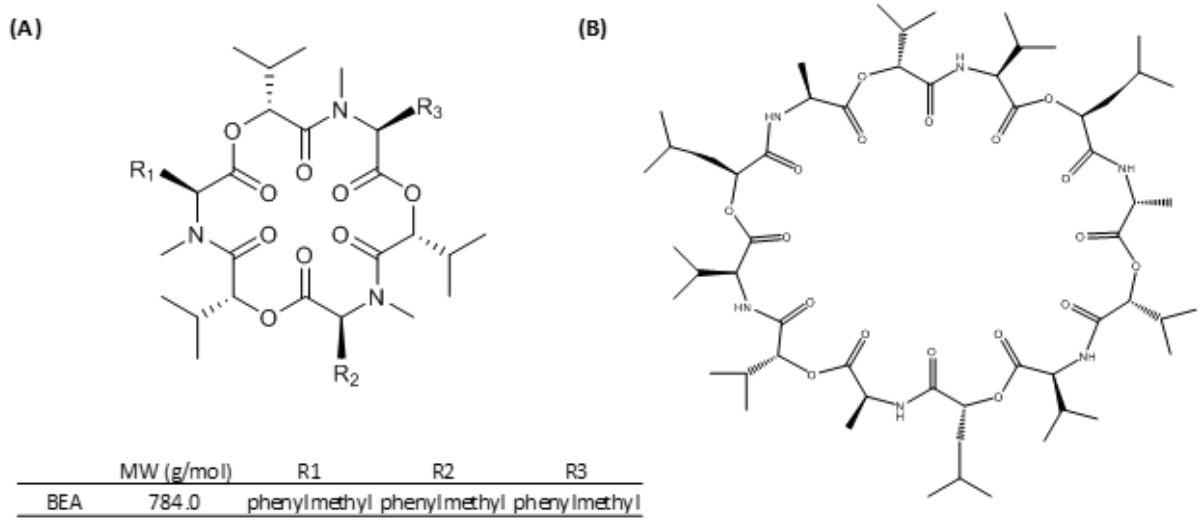
Figures

Figure 1 The chemical structures of beauvericin and enniatins (A) and cereulide (B)



	MW (g/mol)	R1	R2	R3
BEA	784.0	phenylmethyl	phenylmethyl	phenylmethyl
ENN A	681.9	sec-butyl	sec-butyl	sec-butyl
ENN A1	667.9	sec-butyl	sec-butyl	iso-propyl
ENN B	639.8	iso-propyl	iso-propyl	iso-propyl
ENN B1	653.9	iso-propyl	iso-propyl	sec-butyl

Figure 2 Total ion chromatograms of the analytes of a spiked rice sample at 100 µg.kg^{-1}

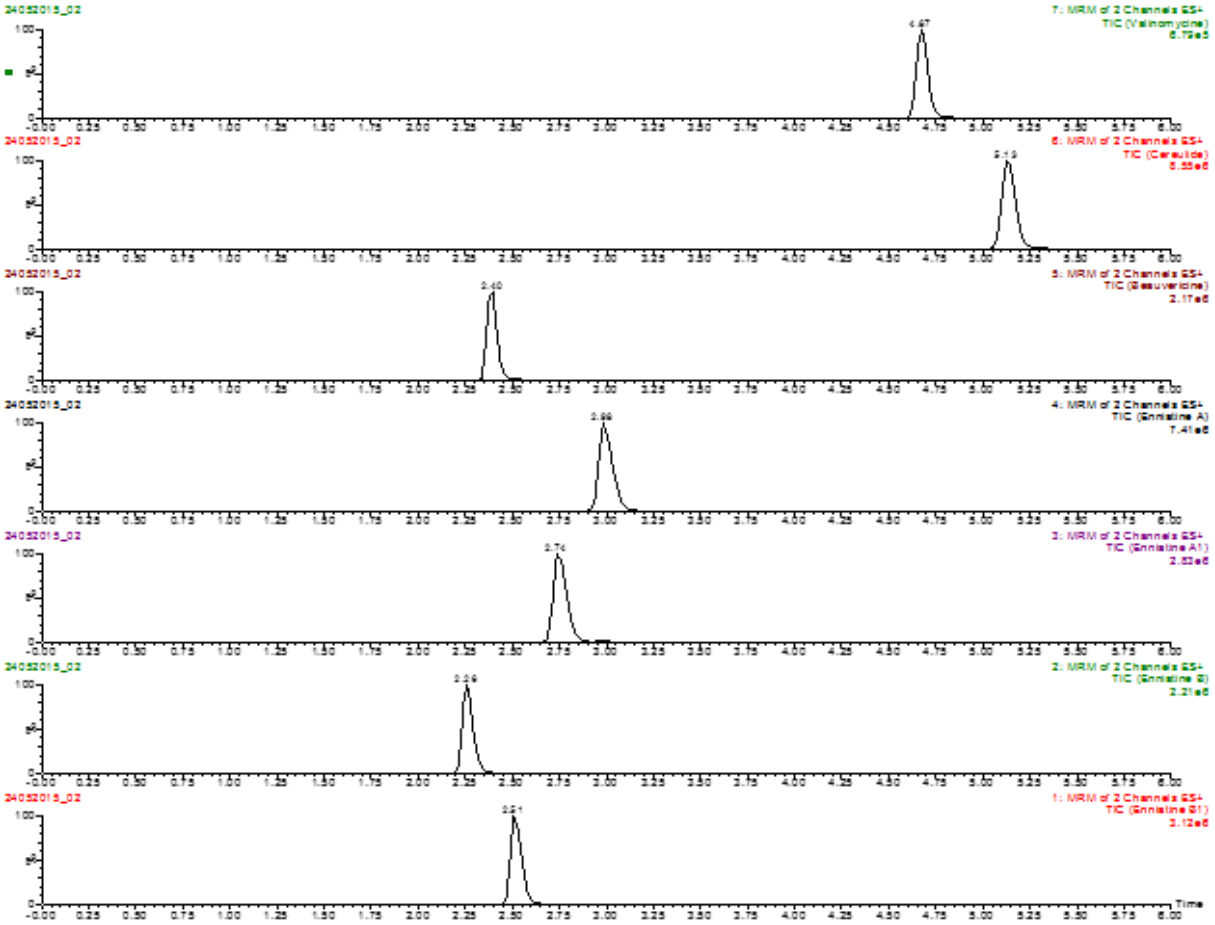
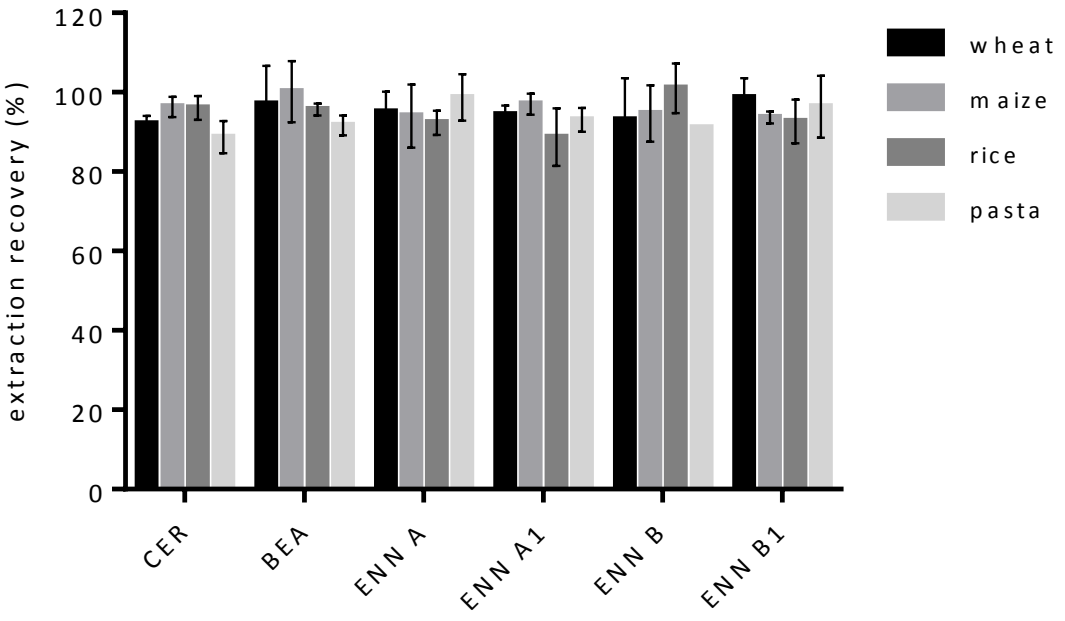

240522015_02
7: MRM of 2 Channels ES+
TIC (Valinomycine)
6.79e5
4.47
240522015_02
6: MRM of 2 Channels ES+
TIC (Cereulide)
5.55e6
5.13
240522015_02
5: MRM of 2 Channels ES+
TIC (Beauvericine)
2.17e6
3.40
240522015_02
4: MRM of 2 Channels ES+
TIC (Enniatine A)
7.41e6
3.96
240522015_02
3: MRM of 2 Channels ES+
TIC (Enniatine A1)
2.53e6
3.74
240522015_02
2: MRM of 2 Channels ES+
TIC (Enniatine B)
2.21e6
3.39
240522015_02
1: MRM of 2 Channels ES+
TIC (Enniatine B1)
3.12e6
3.61
Time

Figure 3 Mean extraction recovery of CER, BEA and ENNs (%) (n=3) in wheat, maize, rice and pasta



Tables

Table 1 Optimized ESI$^+$ MS/MS parameters for all analytes, including valinomycin.

Compound	Precursor ion (m/z)	Molecular ion	Product ion (m/z)	Cone (V)	Collision (eV)
Cereulide	1170.7	$[M + NH_4]^+$	172.3*	70	76
			314.2	70	62
Beauvericin	801.3	$[M + NH_4]^+$	244.3*	38	47
			262.4	38	47
Enniatin A	699.2	$[M + NH_4]^+$	210.3	20	43
			682.3*	20	17
Enniatin A1	685.4	$[M + NH_4]^+$	210.3*	38	32
			228.3	38	36
Enniatin B	657.3	$[M + NH_4]^+$	196.3	40	30
			640.2*	40	17
Enniatin B1	671.2	$[M + NH_4]^+$	196.3	32	32
			654.0*	32	18
Valinomycin[a]	1128.6	$[M + NH_4]^+$	343.5*	66	62
			713.5	66	44

[a] Valinomycin was used as internal standard

Table 2 Concentration range (µg.kg^{-1}) and R^2 values of the matrix-matched calibration curves in wheat, maize, rice and pasta, with the corresponding LOD and LOQ (µg.kg^{-1})

	Wheat				Maize				Rice				Pasta			
	Range	R^2	LOD	LOQ	Range	R^2	LOD	LOQ	Range	R^2	LOD	LOQ	Range	R^2	LOD	LOQ
CER	2-400	0.995	1.0	2.9	2-400	0.996	0.1	0.3	2-400	0.997	0.1	0.3	1-100	0.989	0.2	0.7
BEA	2-400	0.983	0.6	1.9	2-400	0.988	0.1	0.3	2-400	0.996	0.2	0.7	1-100	0.986	0.3	1.0
ENN A	2-200	0.983	0.7	2.2	2-200	0.984	0.5	0.8	2-200	0.990	0.9	0.4	1-100	0.989	0.2	0.6
ENN A1	2-400	0.998	0.5	1.5	2-400	0.994	0.5	1.4	2-400	0.995	0.9	2.6	1-100	0.995	0.5	1.4
ENN B	2-400	0.980	0.7	2.0	2-400	0.997	0.2	0.6	2-400	0.995	0.2	0.7	1-100	0.983	0.3	1.0
ENN B1	2-400	0.978	0.8	2.4	2-400	0.992	0.5	1.5	2-400	0.988	0.3	0.9	1-100	0.980	0.4	1.2

Table 3 Results for trueness expressed as apparent recovery (R_{app}), repeatability (RSD_r), intermediate precision (RSD_R), and expanded measurement uncertainty (U) for all analytes on low, medium and high concentration level in wheat, maize, rice and pasta

	Wheat					Maize					Rice					Pasta				
	conc ($\mu g.kg^{-1}$)	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	conc ($\mu g.kg^{-1}$)	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	conc ($\mu g.kg^{-1}$)	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	conc ($\mu g.kg^{-1}$)	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)
CER	2	101	7.0	14	37	2	100	8.2	13	26	2	92	9.8	14	27	1	14	19	19	38
	200	97	2.5	2.2	10	200	103	6.0	8.1	16	200	103	5.6	9.1	18	50	34	7.1	10	20
	400	101	3.1	4.9	14	400	106	4.8	5.6	11	400	105	8.0	8.0	16	100	58	6.8	6.8	14
BEA	2	99	9.1	13	32	2	101	15	15	29	2	98	17	17	33	1	17	14	19	38
	200	93	3.9	6.6	23	200	104	6.8	6.8	14	200	104	3.1	9.4	19	50	34	7.1	7.1	14
	400	85	2.4	2.4	11	400	94	7.0	9.2	18	400	91	5.3	8.2	17	100	58	5.4	5.5	11
ENN A	2	97	6.2	7.6	22	2	98	19	19	37	2	93	9.1	9.7	20	1	10	8.5	8.9	18
	100	94	6.8	11	34	100	100	5.6	7.2	14	100	84	10	12	25	50	37	5.6	5.6	11
	200	92	4.4	7.2	25	200	99	7.7	8.2	16	200	99	12	12	24	100	62	4.9	4.9	9.8
ENN A1	2	101	17	18	49	2	99	9.0	13	27	2	87	12	12	24	1	13	14	15	29
	200	99	4.5	8.7	24	200	108	5.3	5.3	11	200	101	3.7	8.7	17	50	34	5.3	5.3	11
	400	100	4.8	13	35	400	108	3.6	4.7	9.3	400	100	7.2	13	25	100	63	2.5	4.4	8.7
ENN B	2	95	15	21	47	2	98	9.1	20	39	2	102	15	16	33	1	17	5.3	17	33
	200	100	4.4	6.7	19	200	104	5.5	6.6	13	200	101	15	16	32	50	41	7.8	7.9	16
	400	102	6.0	7.4	24	400	108	2.6	5.2	10	400	104	6.0	7.3	15	100	57	9.1	11	22
ENN B1	2	97	4.2	6.0	36	2	108	20	20	40	2	97	9.8	13	27	1	14	17	18	37
	200	104	3.6	6.2	22	200	109	4.5	8.6	17	200	108	5.1	11	23	50	36	6.9	9.1	18
	400	90	6.7	8.7	39	400	98	1.7	2.8	5.6	400	94	4.4	5.9	12	100	56	5.7	6.5	13

Table 4 Presence of cereulide, beauvericin and enniatins in wheat, maize, rice and pasta (n.d. = not detected).

Samples	CER		BEA		ENN A		ENN A1		ENN B		ENN B1	
	positive samples (%)	maximum level ($\mu g.kg^{-1}$)	positive samples (%)	maximum level ($\mu g.kg^{-1}$)	positive samples (%)	maximum level ($\mu g.kg^{-1}$)	positive samples (%)	maximum level ($\mu g.kg^{-1}$)	positive samples (%)	maximum level ($\mu g.kg^{-1}$)	positive samples (%)	maximum level ($\mu g.kg^{-1}$)
Wheat (n=10)	-	n.d.	-	n.d.	-	n.d.	20	14.6 ± 3.9	70	89.2 ± 16.5	70	42.5 ± 15.4
Maize (n=23)	-	n.d.	74	209.0 ± 39.7	-	n.d.	9	10.7 ± 2.4	17	195.5 ± 47.0	9	40.7 ± 12.3
Rice (n=12)	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
Pasta (n = 12)	-	n.d.	-	n.d.	-	n.d.	-	n.d.	75	99.2 ± 38.8	50	21.0 ± 7.8