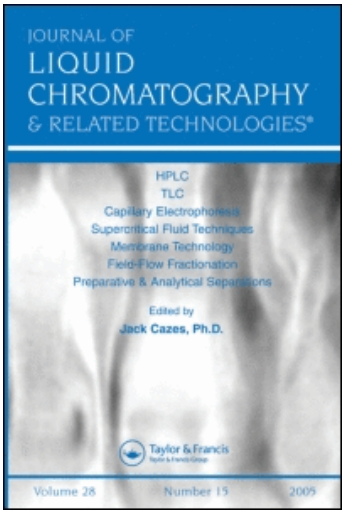

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Determination of *Fusarium*-Mycotoxins Beauvericin and Enniatins with Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Abstract: A liquid chromatography–mass spectrometric method was developed and validated to determine *Fusarium* mycotoxins beauvericin and enniatins (A, A_1, B, B_1) in grain samples. For the first time, a triple quadrupole mass spectrometer with multiple reaction monitoring was used for the analysis of these mycotoxins, which allowed the simultaneous structural identification of the analytes. The sample preparation involved direct purification of sample extracts with reversed-phase–solid phase extraction, enabling the avoidance of time consuming and loss-causing liquid–liquid extractions.

The method validation included the determination of selectivity, repeatability, limit of detection, limit of quantification, recovery, and linearity. The developed method proved to be sensitive and repeatable for the analysis of the mycotoxins in grain matrix. Since the method was originally developed solely for beauvericin, the recoveries of some enniatins remained rather low, except at the lowest spiking level. This was due to the natural contamination of grain matrix with enniatins B and B_1, which resulted in higher recoveries for these mycotoxins. The mean recoveries for beauvericin and enniatins A, A_1, B, and B_1 were 76–82%, 55–66%, 71–80%, 57–103%, and 68–116%, respectively. The calculated limits of quantification for beauvericin and enniatins A, A_1, B, and B_1 were 0.2, 0.2, 0.7, 0.9, and 1.5 μg/kg, respectively.

Keywords: Beauvericin, enniatins, LC–MS, validation

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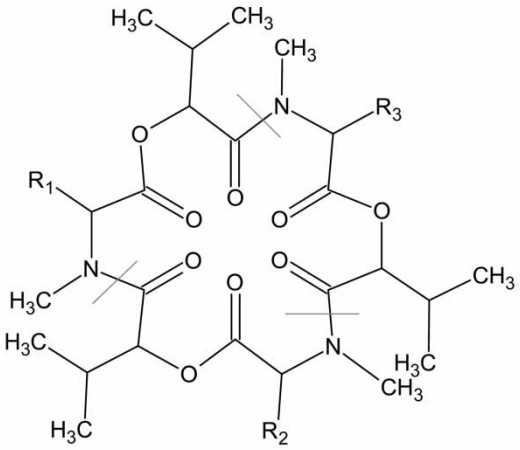
INTRODUCTION

Beauvericin and enniatins A, A_1, B, and B_1 are mycotoxins produced by different *Fusarium* species such as *F. verticillioides, F. proliferatum, F. subglutinans, F. oxysporum, F. poae*, and *F. avenaceum*,[1–3] which are known to be able to colonise many commercially important food commodities around the world. Beauvericin and enniatins are cyclic hexadepsipeptides consisting of alternating amino acid residues.[4] The *N*-methylamino acid substitution in beauvericin is a phenylmethyl residue and enniatins have *iso*-propyl and *sec*-butyl moieties.[5] The structures of beauvericin and enniatins are presented in Figure 1.

Beauvericin and enniatins are efficient ionophoric compounds; in other words, they are capable of transferring monovalent and divalent cations through cell membranes.[6] Their toxicity has not been fully evaluated,



	R1	R2	R3
Beauvericin	Phenylmethyl	Phenylmethyl	Phenylmethyl
Enniatin A	*sec*-butyl	*sec*-butyl	*sec*-butyl
Enniatin A1	*sec*-butyl	*sec*-butyl	*iso*-propyl
Enniatin B	*iso*-propyl	*iso*-propyl	*iso*-propyl
Enniatin B1	*iso*-propyl	*iso*-propyl	*sec*-butyl

Figure 1. The structures of beauvericin and enniatins. The marked bonds indicate the dissociation sites of the molecular ions during a collision induced fragmentation in MS/MS (see section on LC–MS/MS analysis).

although there are some reports that these compounds possess cytotoxic[7] and insecticidal[8] properties.

A number of methods have been developed to determine beauvericin and/or enniatins in various matrices.[2,9–14] The methods include the common steps applied in mycotoxin analyses: sample preparation using extraction of the analytes and removal of impurities using different types of columns. Sample analysis is carried out using high performance liquid chromatography (HPLC) with ultraviolet (UV)[2,9,10,13,14] or mass spectrometric (MS) detection.[11,12] However, many of the published methods are laborious and time consuming and suffer some other limitations, such as the requirement for low detection wavelengths, if UV detection is applied. This is a clear disadvantage if complex sample matrices are to be analysed. To be able to evaluate the possible chronic toxic effects of mycotoxins, one must be able to detect the minute concentrations of these mycotoxins found in natural samples and, thus, more sensitive methods need to be developed.

In this article, we report the development and validation of a simple, feasible, and sensitive method for analysing beauvericin and enniatins in grain samples. For the first time, a triple quadrupole MS with multiple reaction monitoring (MRM) technique has been used for the analysis of beauvericin and enniatins.

EXPERIMENTAL

Standards

Mycotoxin standards (beauvericin and enniatins $A + A_1 + B + B_1$) were purchased from Sigma (St. Louis, MO, USA). The standard solutions of beauvericin (1–100 μg/mL) and enniatins (2–200 μg/mL) were prepared in acetonitrile.

Chemicals

Acetonitrile, methanol, potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), sodium chloride (NaCl) and ammonium acetate (CH_3COONH_4) were purchased from J.T. Baker (Deventer, Holland). The water used was milli-Q water, purified with a Millipore Milli-Q Plus system (Millipore, Espoo, Finland).

Sample Preparation

Samples were ground with a laboratory mill (Bamix, Mettlen, Switzerland). A total of 25 g of ground cereal mixture (wheat/rye/barley, 3 : 2 : 1, w/w/w)

was spiked with different concentrations of mycotoxin standard solutions and extracted with 100 mL of 84% acetonitrile in water for 2 h using a VKS-75 horizontal shaker (Edmund Bühler, Bodelshansen, Germany) at room temperature. The extracted samples were filtered through an S&S 602 H 1/2 filter paper (Schleicher & Schuell, Dassal, Germany). The filtered extracts were stored at $+4^{\circ}$C, no longer than 1 month, until analysts. One ground Finnish barley sample was also analysed for the presence of beauvericin and enniatins to prove the applicability of the method to detect beauvericin and enniatins in naturally contaminated samples.

Sample extracts were purified using solid phase extraction (SPE) columns Bond Elut C_8, 200 mg (Varian Inc. EA Middelburg, The Netherlands). The purification column was conditioned with 3 mL of methanol followed by 3 mL of 0.5 M phosphate buffer (pH 7.0). The sample extract (5 mL) was applied into the column after dilution with 5 mL of phosphate buffer. The column was washed with 3 mL of 60% acetonitrile in water and the analytes were eluted from the column with 5 mL of 100% acetonitrile. The eluate was evaporated to dryness using nitrogen flow and redissolved with HPLC-mobile phase (250 μL of acetonitrile/methanol/0.01 M ammonium acetate, 45 : 45 : 10 v/v/v) and mixed thoroughly with a Vortex-Genie 2 test tube mixer (Scientific Industries Inc., Bohemia, NY, USA). The dissolved sample was filtered through a 0.2 μm syringe filter (Pall Gelman Sciences, Ann Arbor, MI, USA).

LC–MS/MS Analysis

Beauvericin and enniatins were analysed with a Waters Alliance 2695 liquid chromatograph (Waters Co., Milford, MA, USA) connected to a MicroMass Quattro Micro triple-quadrupole MS (MicroMass Ltd., Manchester, UK). An electrospray ionisation (ESI) probe was used in the analyses. The analytical column was a Luna C_{18} (5 μm) 150 × 3.00 mm (Phenomenex Inc., Torrance, CA, USA). An aliquot of 10 μL of the sample was injected into the analytical column. An isocratic elution was used for the HPLC analysis, in which the composition of the eluate was acetonitrile/methanol/ammonium acetate (0.01 M, pH 7) in a ratio of 45 : 45 : 10 (v/v/v). The flow rate of the mobile phase was 0.5 mL/min and the chromatographic run time was 8 min. A positive ionisation mode was used with the ESI-probe. The parameters of the MS were optimized using the standard solutions. The best response was recorded with the following parameters: cone voltage 45 V, capillary voltage 3.80 kV, source temperature 150°C and desolvation temperature 270°C. Argon (AGA, Finland) was used as a collision gas.

The MRM technique was used for identification and quantification, in which protonated molecules $[M + H]^+$ of the analytes (beauvericin m/z 784, enniatin A m/z 682, enniatin A_1 m/z 668, enniatin B m/z 640, enniatin B_1 m/z 654) were

fragmented in the collision cell to the product ions (beauvericin m/z 244 and 262, enniatin A m/z 210 and 228, enniatin A_1 m/z 210 and 228, enniatin B m/z 196 and 214, enniatin B_1 m/z 196 and 214) with the collision gas energy of 25 eV. Beauvericin and enniatins are cyclic depsipeptides containing three residues (Fig. 1); their fragmentation yields characteristic monomer product ions. Sewram et al.[12] reported the fragmentation of beauvericin to be due to the cleavage of the amide bond. The product ions detected in our method were specific protonated monomers of the molecular ions or the product ions obtained from the loss of water in the monomer fragments. The product ions observed for beauvericin and enniatins under MRM experiments are presented in Table 1. The product ions were each monitored for a dwell time of 0.40 sec and an inter-channel delay of 0.05 sec. The calibration curves were achieved via external standards by injecting standard mixtures dissolved into the mobile phase at six different concentrations (beauvericin 10–300 μg/kg; enniatin A 0.6–18 μg/kg; enniatin A_1 4–120 μg/kg; enniatin B 3.8–114 μg/kg; enniatin B_1 10.8–324 μg/kg). The retention factors (k') of beauvericin, enniatins A, A1, B and B1 were 1.72, 2.81, 2.32, 1.89, and 1.55, respectively.

Validation of the Method

The method validation included the determination of selectivity, repeatability, limit of detection (LOD), limit of quantification (LOQ), recovery, and linearity. Six replicates of spiked samples, at three concentration levels (spiking level 1: beauvericin 10 μg/kg, enniatin A 0.6 μg/kg, enniatin A_1 4 μg/kg, enniatin B 3.8 μg/kg, enniatin B_1 10.8 μg/kg; spiking level 2: beauvericin 50 μg/kg, enniatin A 3 μg/kg, enniatin A_1 20 μg/kg, enniatin B

Table 1. The fragment ions observed for beauvericin and enniatins

Analyte	m/z of fragment ion observed	Interpretation
Beauvericin	262	$[\text{Monomer} + \text{H}]^+$
	244	$[\text{Monomer} + \text{H} - \text{H}_2\text{O}]^+$
Enniatin A	228	$[\text{Monomer} + \text{H}]^+$
	210	$[\text{Monomer} + \text{H} - \text{H}_2\text{O}]^+$
Enniatin A1	228	[Monomer with *sec*-butyl residue + H]$^+$
	210	[Monomer with *sec*-butyl residue + H − H_2O]$^+$
Enniatin B	214	$[\text{Monomer} + \text{H}]^+$
	196	$[\text{Monomer} + \text{H} - \text{H}_2\text{O}]^+$
Enniatin B1	214	[Monomer with *iso*-propyl residue + H]$^+$
	196	[Monomer with *iso*-propyl residue + H − H_2O]$^+$

For fragmentation, also see Figure 1.

19 μg/kg, enniatin B_1 54 μg/kg; spiking level 3: beauvericin 100 μg/kg, enniatin A 6 μg/kg, enniatin A_1 40 μg/kg, enniatin B 38 μg/kg, enniatin B_1 108 μg/kg) and calibration curves with and without matrix were analysed on four different days. The calibration curves with matrix were prepared at the same six levels as the calibration curves without matrix (see section on LC–MS/MS Analysis). The grain matrix was spiked with corresponding amounts of standards and prepared as regular samples.

RESULTS AND DISCUSSION

Development of SPE Purification for Beauvericin

The structure of beauvericin has polar and non-polar properties, a fact which influences its solubility. However, beauvericin is naturally hydrophobic and is insoluble in water.[15] Several solvents or solvent mixtures (100% methanol; acetonitrile/methanol/water 16 : 3 : 1; 55% methanol; 55% methanol +1% NaCl; 60–90% acetonitrile) were tested in the extraction of beauvericin in grain samples. As the sample purification step was still not developed, after extraction, the samples were purified with silica-SPE columns and analysed with HPLC–UV as described by Krska et al.[9] The best recovery of beauvericin was achieved using 84% acetonitrile in water (data not shown), which was chosen as an extraction solvent.

The published beauvericin methods are often time consuming and, in most cases, include loss-causing liquid–liquid extractions and evaporation steps. By utilizing the modern technological advantages provided by SPE, these shortcomings can be overcome. Due to the properties of the analyte, we decided to use a C_8-phase instead of a C_{18}-phase. The C_8-phase allows good retention of the analytes but does not necessitate the use of high volumes of organic elution solvent. Also, the retention of unwanted impurities is probably lower in the C_8-than in the C_{18}-phase.

Beauvericin includes three nitrogen atoms and, thus, the pH of the eluent may play a significant role on the retention behaviour of the molecule in the reversed-phase analysis, although the main mechanism for retention in the C_8-phase is hydrophobic attraction. Beauvericin does not have any titrable (ionic) groups,[15] but we suggest that, at high pH, the retention of the analyte is better, due to the uncharged form of the polar groups. This is because carbonyl- and methyl-substituted nitrogen groups of beauvericin molecule have free electron pairs that can act as nucleophiles. To assess this hypothesis, the effect of five different pH values (pH 3.0, 5.0, 7.0, 9.0, and 10.6) of standard buffer solutions and water were tested for the retention of beauvericin in the C_8-SPE columns (Figure 2).

When the sample was introduced into the column with a phosphate buffer at pH 7.0, the recovery of beauvericin was about 100%. The low

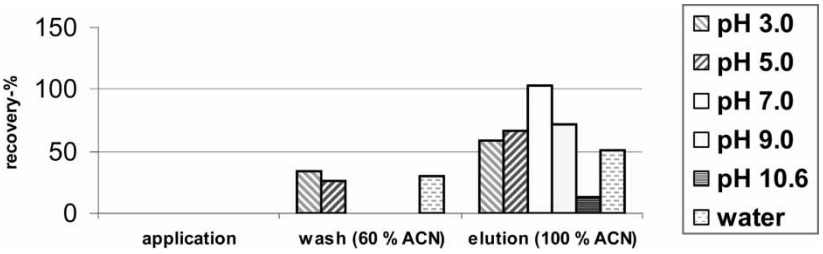


Figure 2. The retention of beauvericin in C_8-SPE column. Before application, the sample extracts were diluted with standard buffer solutions at various pHs.

recovery seen at pH 10.6 is due to the breakdown of the silica-based sorbent under such basic conditions. The use of water results in a low recovery and, if it is compared to the recovery with phosphate buffer at pH 7.0, it can be concluded that potassium phosphate-ions must have an effect on the retention behaviour of the analyte in C_8-phase.

Development of the LC–MS/MS Method

Beauvericin and enniatins are ionophoric compounds that can form complexes with mono- and divalent cations, such as Na^+, K^+, and Ca^{2+} by weak interactions with the carbonyl-groups orienting to the middle of the molecule.[16] They are not very selective with respect to the actual cations because of the flexible structure of the molecule. Mono- and divalent cations exist as contaminants in nearly all solvents, instrument linings, etc. If the complex-formation capability of beauvericin and enniatins is not controlled in some way, all of the different complexes ($M + Na^+$, $M + K^+$, $M + Ca^{2+}$, and $M + NH_4^+$) can be present in an LC–MS analysis (Figure 3a). Our findings support the study of Ngoka et al.,[17] in which it was noted that different complexes are fragmented in various ways, and this can lead to problems in quantification. By using ammonium acetate in the mobile phase, we could exclude all other complexes for beauvericin and enniatins, except $M + NH_4$ (Figure 3b).

The cone voltage of the MS-ion source also has an effect on the abundance of the different complexes. By adjusting the cone voltage used, we could decrease the relative abundance of ammonium complex, compared to the abundance of the protonated molecule (Fig. 3c). This further increased the reliability of quantification. In MS trace analysis, most often selected ion recording (SIR) or MRM modes are used, which means that only one or two specific ions produced by the analytes are detected. By controlling the formation of complexes via the composition of the mobile

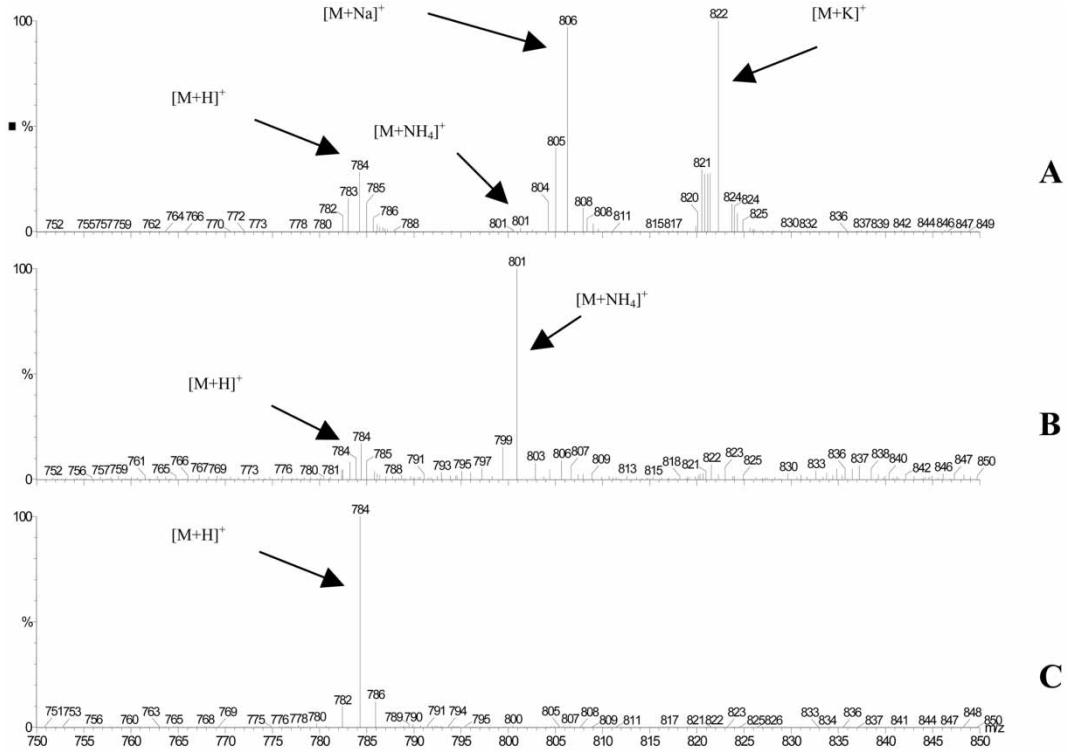


Figure 3. The mass spectrum of beauvericin without (A) and with (B) ammonium acetate in the mobile phase (cone voltage 20 V) and with ammonium acetate using the cone voltage of 45 V (C).

phase and the abundance of the remaining forms of analytes with the cone voltage, beauvericin and enniatins can be quantitated very reliably in samples.

Validation of the Method

Selectivity is the ability of the method to distinguish the response of the analyte from all other responses, for example, the ones originating from the sample matrix compounds. Selectivity of the developed method was tested by comparing the slopes of the six-point calibration curves obtained, with and without matrix (for calibration curve ranges see section on LC–MS/MS Analysis). No statistically significant differences ($p > 0.05$, 2-sided *t*-test) in the slopes of a non-matrix and matrix-assisted calibration curves were observed for beauvericin or enniatins. For this reason, the external calibration without any matrix can be used for the quantification of the analytes.

The acceptable linearity of each point of the calibration curves without matrix was tested with the method of van Trijp and Roos.[18] A tolerance of $100 \pm 10\%$ was accepted for the separate calibration points for good linearity. On that basis, the method can be considered as being linear for beauvericin and enniatins on the ranges tested (see section on LC–MS/MS Analysis).

LOD and LOQ for beauvericin and enniatins were calculated from the responses of the MRM traces for the reagent blank ($n = 20$). LODs (reagent blank mean response $+3 \times$ standard deviation) for beauvericin, enniatin A, A_1, B, and B_1 were 0.1, 0.1, 0.3, 0.4, and 0.7 μg/kg, respectively. The corresponding LOQs (reagent blank mean response $+10 \times$ standard deviation) for the analytes, in turn, were 0.2, 0.2, 0.7, 0.9, and 1.5 μg/kg, respectively. Although lower concentrations of the analytes could be quantified with the method, for practical reasons, however, the LOQs used for the analytes were the lowest points of the calibration curves (see section on LC–MS/MS

Table 2. The mean recoveries of beauvericin and enniatins at three different spiking levels

	Spiking level 1 (μg/kg)	Recovery% (sp. level 1; $n = 24$)	Spiking level 2 (μg/kg)	Recovery% (sp. level 2; $n = 24$)	Spiking level 3 (μg/kg)	Recovery% (sp. level 3; $n = 24$)
Beauvericin	10	82 ± 12	50	81 ± 5	100	76 ± 5
Enniatin A	0.6	59 ± 13	3	66 ± 14	6	55 ± 15
Enniatin A1	4	80 ± 9	20	71 ± 12	40	72 ± 6
Enniatin B	3.8	103 ± 3	19	64 ± 10	38	57 ± 6
Enniatin B1	10.8	116 ± 14	54	73 ± 8	108	68 ± 10

As no matrix effect was observed for the analytes, external quantification was used.

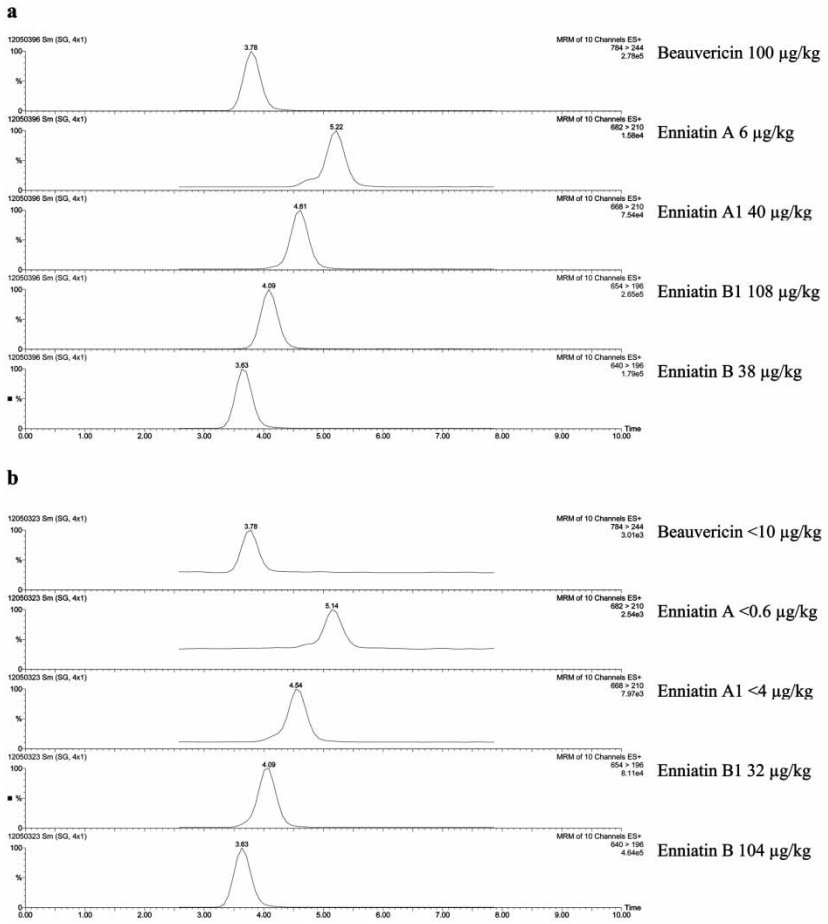


Figure 4. The extracted ion chromatograms of beauvericin and enniatins of a spiked grain sample (a) and of a naturally contaminated grain sample (b). Please refer to Fig. 1 for the chemical structure of the particular analyte.

Analysis). The developed method was proven to be more sensitive than the existing UV methods[2,9,10,13,14] and LC–MS methods,[11,12] where the LODs vary from 0.5 to 1000 µg/kg for beauvericin. It is also noteworthy that enniatin A could be detected using our method, which is not the case with most of the existing UV methods. The commercial standard mixture of enniatins contains only 3% of enniatin A, and the concentration is usually too low to permit UV detection.

The mean recoveries of six replicates on four separate days at three different spiking levels of the analytes are presented in Table 2. Since the

method was originally developed solely for beauvericin determination, and only recently extended to enniatins, the mean recoveries of some of the enniatins may be considered as rather low. However, the method is repeatable, as demonstrated by the standard deviations of the mean recoveries (Table 2). No blank material for enniatin B and enniatin B_1 could be found in Finland because all of the grain samples (wheat, rye, barley, and oats) analysed so far were determined to be contaminated. For this reason, the amount of enniatin B and enniatin B_1 in the matrix was taken into account when we calculated the mean recoveries of the analytes. The presence of the enniatin B and enniatin B1 as natural contaminants in the matrix used, however, resulted in artefactually high recoveries for these mycotoxins at the low spiking level (Table 2).

Examples of the extracted ion chromatograms of analytes of a spiked grain sample and naturally contaminated sample are presented in Figure 4a and b, respectively. Good resolution was achieved for all compounds with the mobile phase used, even though there was a relatively short run time of only 8 min.

In conclusion, a new LC–MS/MS method, with a triple quadrupole mass spectrometer, was developed for the determination of beauvericin and enniatins. Due to the direct application of a crude extract to the RP-SPE column, the problematic, time consuming, and loss-causing liquid–liquid extractions could be avoided. We were able to measure enniatin A with the method, which is not the case in most of the existing methods. LOD and LOQ values obtained demonstrate that the method is more sensitive than the published analytical methods for beauvericin and enniatins. The validation data also indicate that the method is reliable for the determination of beauvericin and enniatins in a grain matrix. Besides the sensitivity and reliability, the MRM method allows, also, the simultaneous structural identification of the analytes for confirmatory purposes.

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