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Determination of the *Fusarium* mycotoxin beauvericin at μg/kg levels in corn by high-performance liquid chromatography with diode-array detection

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Abstract

A method is described for the detection of the *Fusarium* mycotoxin beauvericin (BEA) in corn and corn meal. Spectral data obtained with a diode-array detector showed that the most sensitive wavelength for the detection of BEA is 192 nm. The detection limit for BEA was 50 μg/kg, which is an increase in sensitivity by a factor of at least twenty compared to previously published analytical methods for this mycotoxin.

Keywords: Fusarium; Mycotoxin; Beauvericin

1. Introduction

Fusarium species constitute an important group of fungi, which are known to produce toxic secondary metabolites [1]. The most important mycotoxins known to be produced by *Fusarium spp.* are the large group of trichothecenes, zearalenone, moniliformin, fusarin C and the fumonisins. Another important class of biologically active metabolites from *Fusarium spp.* are cyclodepsipeptides. These compounds contain both amide and ester bonds in alternating sequence and have been shown to be produced by numerous bacteria and fungi [2]. In 1991 Gupta et al. [3] reported the in vitro production of the cyclodepsipeptide toxin beauvericin (BEA) by *Fusarium semitectum* and *F. subglutinans*. More recently, Logrieco et al. [4] reported the production of BEA by isolates of *F. subglutinans* from Peruvian corn at levels up to 250 mg/kg [4] and the natural occurrence of BEA in preharvest *F. subglutinans*-infected corn from Poland (up to 60 mg/kg) [5]. Since then several papers reported the natural occurrence of BEA in corn and the ability of various isolates of *Fusarium* strains from corn to produce BEA [6–9].

BEA (Fig. 1) contains three D-α-hydroxyisovaleryl and three N-methyl-L-phenylalanyl residues in alternating sequence. It is structurally similar to the enniatins, which are also produced by a number of *Fusarium* species, and differs from them only in the nature of the N-methylamino acid [10]. Both enniatins and BEA have been shown to act as ionophores by increasing the permeability of biological and artificial membranes for alkali cations [11].

Although the toxicity of BEA has not been

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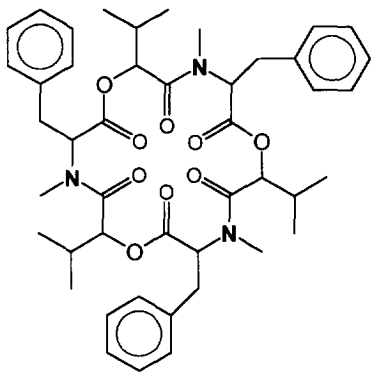

Fig. 1. Idealized drawing of BEA structure with its threefold symmetry. Three of the carbonyl oxygen atoms are pointing above the page and three are pointing below the page.

sufficiently investigated, there are reports on its cytotoxic [12] and insecticidal properties [13], which appear to be related to their ionophoric properties and to their capability for forming complexes with alkali metal cations. The only paper dealing with the cytotoxicity of BEA to mammalian cells reports substantial toxicity of BEA to several mammalian cell lines [14], which indicates that BEA may play a role in human and animal pathology through contamination of corn. Moreover, the presence of BEA at significant levels in cultures of *F. subglutinans* and *F. proliferatum* [15] suggests the potential for its presence in naturally contaminated grain samples associated with animal toxicoses. In a previous report [1], the toxicity of *F. subglutinans* isolates in mycotoxicoses could not be attributed solely to moniliformin production, which means that BEA may be involved in these outbreaks as well. The natural occurrence of BEA and the capability of several isolates of *F. subglutinans* [9] from corn to synthesize this toxin suggests that it could be a significant food and feed contaminant. Thus, sensitive analytical methods for BEA in cereal grains are needed in order to assess toxicity and environmental occurrence.

There are only a few publications which deal with analytical methodology for BEA [3–5]. The sample preparations described involve the extraction of BEA with methanol–1% aqueous NaCl (55:45, v/v), ethyl acetate or acetonitrile–water (1:1, v/v) in a blender, defatting with *n*-hexane, further extraction with dichloromethane, clean-up on a silica column and elution of BEA with chloroform–isopropanol (95:5, v/v). Quantification is performed by reversed-phase HPLC using acetonitrile–water (85:15, v/v) [4] or (70:30, v/v) [3] or methanol–water (67:33, v/v) [15] as a mobile phase and a UV detector set at 225 nm [4,8] and 204 nm [15], respectively. However, the detection limits of these current analytical methods for BEA in corn are 1–50 mg/kg and therefore not suitable for trace analysis at μg/kg levels in corn and corn products. In addition, in published analytical works on BEA no diode-array detection (DAD) has been employed for its identification. Therefore, to our knowledge no UV spectrum of BEA has been published so far which could be used to optimize the monitoring wavelength for UV detection after separation by HPLC.

The main goal of the work presented in this paper was the development of a sensitive analytical method for the detection of BEA in corn and corn products in the lower μg/kg range. The method involves HPLC with DAD and was subsequently applied to the analysis of corn naturally contaminated with *F. subglutinans*.

2. Experimental

2.1. Reagents

BEA, available from Sigma (Deisenhofen, Germany, 99% purity), was dissolved in HPLC-grade acetonitrile (Baker, Gross Gerau, Germany)–monodistilled water (9:1, v/v) to obtain BEA standard solutions. Sodium chloride (Baker) and methanol (Merck, Darmstadt, Germany) of analytical grade and monodistilled water were used for preparation of extraction solvents. Dichloromethane (analytical grade) and chloroform [analytical grade, stabilized with 0.75% (v/v) ethanol] were obtained from Baker; *n*-hexane (analytical grade) was from Merck.

2.2. Sample preparation

For the spiking experiments different amounts of a 100 mg/l standard solution of BEA were added to corn kernels or commercially available corn meal that contained no traces of BEA. A 20-g amount of corn sample was extracted in an Ultra Turrax T25

blender with 100 ml of methanol–1% aqueous NaCl (55:45, v/v) for 3 min, then filtered through Whatman No. 1 filter paper and 50 ml were transferred into a separatory funnel. In the literature [4,5] a defatting step with *n*-hexane (2×50 ml) is described as the next step. However, since the results of our preliminary experiments revealed considerable loss of BEA during this defatting step, we modified the subsequent clean-up procedure, which enabled us to leave out the extraction with *n*-hexane. Hence, instead of carrying out this defatting step, we switched directly to the further extraction step with dichloromethane (3×30 ml) that was also described in the literature [3,5]. The dichloromethane extracts were collected, evaporated to dryness on a rotary evaporator (50°C), dissolved and transferred to 4-ml screw-capped vials (Supelco, Bellefonte, PA, USA) with 4×1 ml chloroform–methanol (3:1, v/v). The solution was then evaporated under nitrogen with gentle heating (50°C). The residue was dissolved in 4×250 μl volumes of chloroform and applied to a silica solid-phase extraction (SPE) column (Supelclean LC-Si, 1 ml, Supelco). The column was preconditioned with 2×1 ml of chloroform and after application of the extract was washed with 4×1 ml of chloroform and 2×1 ml of chloroform–methanol (99:1, v/v). The BEA was eluted with 2 ml of chloroform–methanol (98:2, v/v). The eluent was evaporated under nitrogen (50°C) and reconstituted to 250 μl with the mobile phase.

2.3. HPLC conditions

HPLC analysis was carried out using an apparatus from Hewlett-Packard (HP 1090 Series II) equipped with a DAD system. The use of DAD enabled us to find the optimal absorption wavelength for measurements of BEA and to control the purity of the peaks. The column was reversed-phase Vydac C_{18} (250×4.6 mm I.D.). Acetonitrile–water (90:10, v/v) was used as the mobile phase under isocratic conditions, with a flow-rate of 1.2 ml/min. 192 nm was used as the detection wavelength. Spectra were recorded after subtracting the solvent absorption. Injections were made in duplicate and the injection volume was 20 μl. Quantification was done by comparison of the peak height of BEA against a calibration curve of the peak height obtained with authentic standard.

2.4. Identification of beauvericin

Identification was achieved by comparing the retention times and spectral data (obtained by DAD) with the standard obtained from Sigma.

2.5. Statistical analysis

The calibration data were subjected to statistical analysis using the Microsoft Excel macro Validata (Rohrer and Wegscheider, Austria).

3. Results and discussion

3.1. Optimization of the detection wavelength

Fig. 2 shows the UV spectrum of BEA obtained with DAD, with its absorption shoulder at 209 nm. However, maximum absorption occurs at 192 nm. Since the mobile phase used shows only an absorption of 0.42 AU at 192 nm, measuring at this unusual, and rather unspecific wavelength, is possible. In a first set of experiments BEA was simultaneously measured at 192 and 225 nm both in pure mobile phase and in corn matrix, including naturally contaminated corn. Under our experimental conditions a dramatic increase in sensitivity was observed at 192 nm (by a factor of up to 14.6) when compared to the absorbance values measured at 225 nm, the wavelength most frequently found in the literature. Furthermore, there is excellent linear correlation between sample concentration and the height of the peaks in the HPLC profile from 1 to 100 μg/ml of

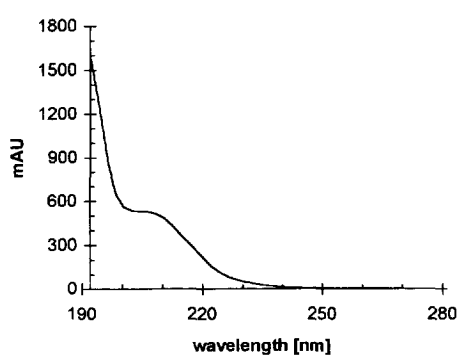

Fig. 2. UV spectrum of BEA obtained with DAD.

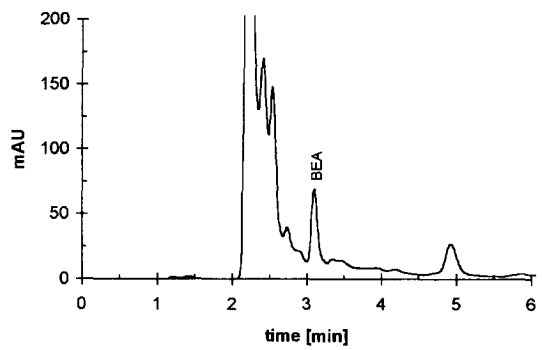

Fig. 3. HPLC chromatogram obtained from corn spiked with 100 μg/kg BEA.

BEA standard (r=0.9998). Fig. 3 shows the HPLC chromatogram obtained for the measurement of 100 μg/kg BEA in corn at 192 nm. In Fig. 4 the chromatogram of an unspiked sample (blank) is overlaid with a chromatogram of a 20 ng/μl BEA standard. This figure clearly demonstrates that there are also no interferences by the remaining matrix components at this wavelength, due to the appropriate sample clean-up.

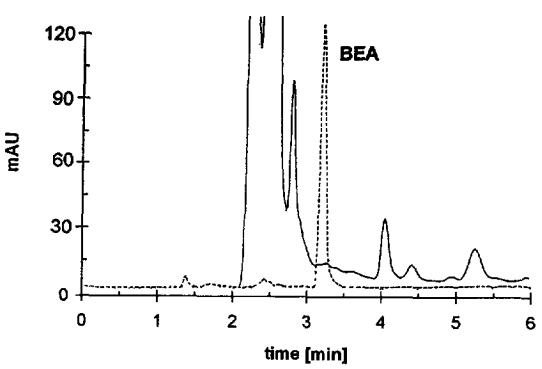

Fig. 4. HPLC chromatogram obtained from an unspiked sample (full line) overlaid with a chromatogram of a 20 ng/μl BEA standard (dotted line).

3.2. Recovery of beauvericin

In a first set of experiments we compared the recovery of our method with state-of-the-art methods. When employing the defatting step with n-hexane [5], the recovery decreased from 78% to 36% (n=2), at a spiking level of 500 μg/kg, and from 86% to 33% (n=2), at a spiking level of 1000 μg/kg. The relative losses of BEA due to this defatting step are therefore 54 and 62%, respectively. The clean-up procedure described here enabled us to leave out the extraction with n-hexane. Thus, the recovery of BEA could be improved by at least 50%. Combined with the use of 192 nm as the detection wavelength, the detection limit for BEA could be improved to 50 μg of BEA per kg of maize sample, which is an increase in sensitivity by a factor of ~20 compared to previously published analytical methods for this mycotoxin.

For the determination of the mean recovery of the method, spiking experiments at four concentration levels (100, 500, 1000 and 2000 μg/kg) were carried out. Table 1 shows the recoveries obtained for BEA added to corn meal. According to these data, the mean recovery for BEA in the concentration range 100–2000 μg/kg was 82.6% for n=8 (S.D.=5.0%).

3.3. Determination of LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the guidelines of the German norm DIN 32645 (May 1994). In this norm, the LOD and LOQ are derived

Table 1
Recovery of BEA added to corn meal

Concentration present (μg/kg)	Concentration found (μg/kg)		Mean recovery (%)
	First analysis	Second analysis	
100	88.6	87.3	88.0
500	376	381	75.7
1000	869	834	85.2
2000	1630	1638	81.7

from the y-intercept and its confidence interval after linear regression. The LOD of 50 μg/kg BEA in corn samples was calculated from the calibration curve obtained from the spiking experiments described above (confidence interval=95%, degrees of freedom=8). The LOQ of 150 μg/kg BEA in corn was obtained with a relative precision of 33%.

3.4. Characterization of the loss of BEA during the analytical procedure

In order to determine the losses of BEA during the analytical procedure, equal amounts of BEA, corresponding to a spiking level of 1000 μg/kg, were added to the sample at four crucial stages of the method. Hence, we spiked (A) the corn before extraction, (B) the filtered extract, (C) the dichloromethane phase after re-extraction of the corn extract and (D) the extract before applying it to the SPE column. For all analyses 20 g of corn blank were used. Table 2 reveals that for an overall recovery of 85.2%, the losses of BEA during the analysis are almost equally distributed, ranging from 2 to 6% for each step. Hence, the optimization of one of these steps alone cannot contribute to a significant improvement in the recovery. Therefore, no further experiments were carried out to improve the recovery of the method.

3.5. Applicability

The analytical method was applied to the analysis of corn from Poland that had been naturally contaminated with *F. subglutinans* and had a BEA content of 2.5 mg/kg. The chromatogram of this sample (Fig. 5) shows that the method is also well suited for the trace analysis of BEA in more complex matrices, such as naturally contaminated corn.

Table 2
Recoveries obtained after addition of BEA at four different stages of the analytical procedure (spiking level: 1000 μg of BEA per kg corn meal)

Spiking	Mean recovery (%) (n=2)
Corn before extraction	85.2
Filtered extract	87.4
Dichloromethane phase after reextraction of corn-extract	93.6
The extract before applying to the SPE column	97.9

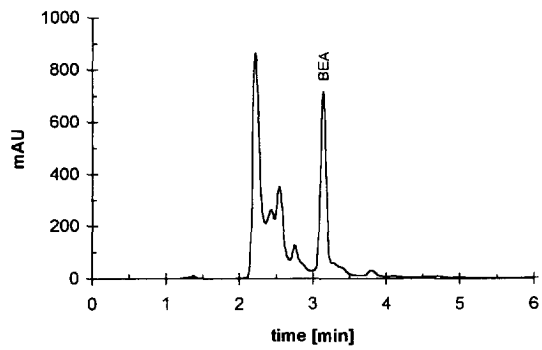

Fig. 5. HPLC chromatogram obtained from corn naturally contaminated with *Fusarium subglutinans* (2.5 mg of BEA/kg).

4. Conclusions

In this work the usefulness of DAD for optimization of the detection wavelength is demonstrated. A detection limit of 50 μg of BEA per kg of corn was reached using the unusual wavelength of 192 nm, combined with an appropriate sample clean-up procedure. This is an increase in sensitivity by a factor of at least 20 compared to previously published methods for the determination of BEA. In addition, the detection limit calculated in this work was obtained in a corn matrix from statistical evaluation of the calibration data, whereas previously published work referred to the analysis of BEA standards without specification of recoveries. The developed method is also well suited for the analysis of BEA at μg/kg levels in corn and corn meal and is therefore suitable for assessing the as yet unknown occurrence of BEA at trace levels in corn.

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