Simultaneous Determination of Beauvericin, Enniatins, and Fusaproliferin by High Performance Liquid Chromatography†

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A rapid, sensitive and inexpensive HPLC method for routine screening of beauvericin, fusaproliferin, and enniatin B_1, A_1, and B has been optimized. Detection limits were determined, ranging between 0.5 and 3.6 ng according to the compound obtained after spiking samples with each mycotoxin at 10−56 μg/mL concentration range; recoveries averaging from 56 to 74% were obtained. LC-MS conditions for enniatin analyses by API electrospray technique were set up, this allowing a unique identification of three different enniatins.

Keywords: *Beauvericin; enniatin; fusaproliferin; HPLC; Fusarium*

INTRODUCTION

Mycotoxins, constituted by a heterogeneous group of toxic substances produced by fungi, have a diverse array of biological effects in humans and animals such as carcinogenicity, teratogenicity and mutagenicity (Arai and Hibino, 1983; Busby and Wogan, 1984; Schlatter et al., 1996; Ritieni et al., 1997a). The presence of mycotoxins in food and feeds is a high potential risk to human and animal health. In addition, they have a significant economic impact since they cause a loss in farm animals and render commodities unacceptable for national and international trade.

Beauvericin (BEA), enniatins (ENNs), and fusaproliferin (FP) are mycotoxins mainly produced by several fungi belonging to *Fusarium* genus. The most important fungi species producing these toxic metabolites and involved in stalk and maize ear rot disease are *F. proliferatum*, *F. subglutinans*, *F. moniliforme*, and *F. avenacum*.

BEA, a cyclo(D-Hyv-*N*-methyl-L-Phe)$_3$ (Figure 1a), is structurally very similar to ENNs (Figure 1b) having *N*-methyl-L-amino acid residues (Strongman et al., 1988). Moreover, the biosynthetic formation pathway of BEA and ENNs, occurring via a thiotemplate mechanism (Peeters et al., 1988; Zocher et al., 1982; Kleinkauf, 1979) are similar, and both of the mycotoxin groups have a 3-fold axis of symmetry and an approximate inversion center. ENNs are divided into three subgroups: type A and B have *N*-methylisoleucine and/or an *N*-methylvaline moiety, while type C, which has three *N*-methylleucine residues, has only been obtained by chemical synthesis (Ovchinnikov et al., 1964). Finally, ENN-A_1, ENN-B_1, and ENN-B have different aliphatic chains lengths.

All these cyclic depsipeptides have been found to be produced by *Fusarium* species belonging to *Liseola* section (Grove and Pople, 1980) mainly *F. subglutinans*

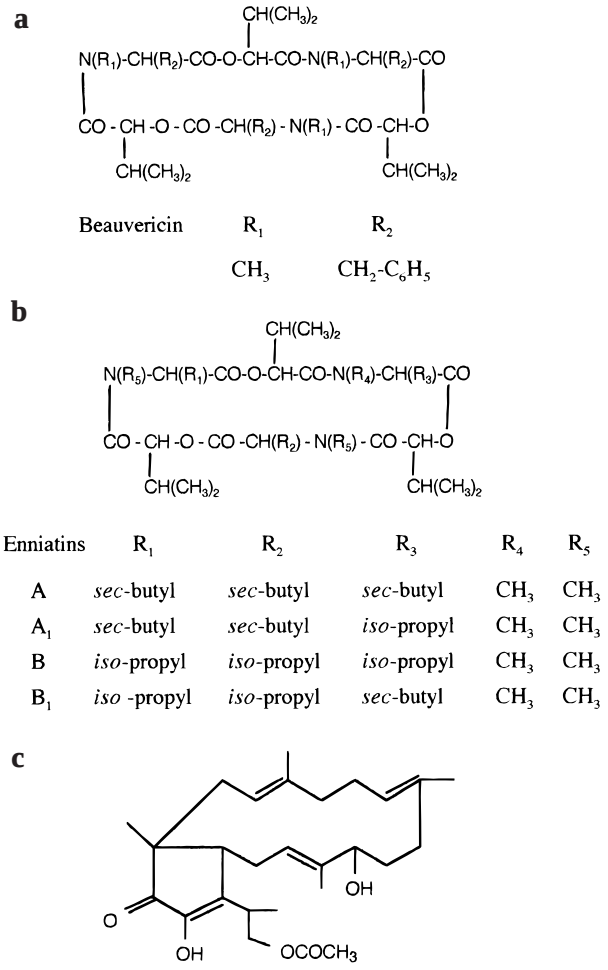

Figure 1. Chemical structure of BEA (a), ENNs (b), and FP (c).

(Logrieco et al., 1991; Kostecki et al., 1995) Nelson, Toussoun, and Marasas (Gupta et al., 1991) and *F. proliferatum* (Matsushima) Nirenberg (Moretti et al., 1994). These cyclodepsipeptides show antimicrobial activity that was ascribed to their capability of translocating cations without any formation of pores through

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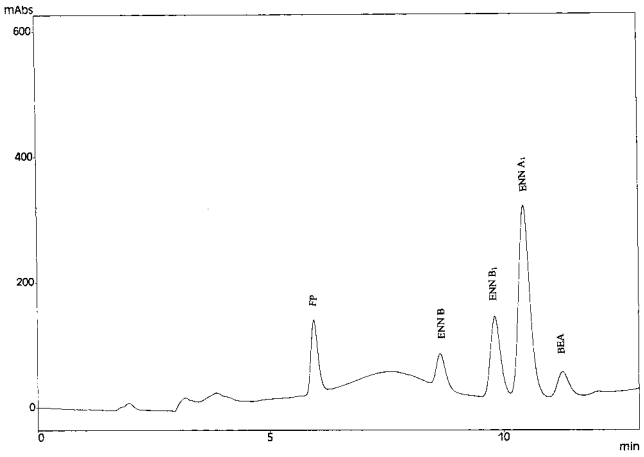

Figure 2. HPLC separation (205 nm) and retention times of standard mixture of mycotoxins: FP (5.94 min), ENN B (8.64 min), ENN B_1 (9.81 min), BEA (10.43 min), ENN A_1 (11.26 min).

the biological and/or artificial bilayer membranes (Prince et al., 1974). They exhibit low water-solubility. Cytotoxicity of BEA toward mammalian cells has been described (Macchia et al., 1995), and recently the interaction of BEA with oligonucleotides monitored by mass spectrometry has been reported (Pocsfalvi et al., 1997). These biological results confirm the risks correlated to BEA and ENN in foodstuffs and their derivatives.

FP has a sesterterpene skeleton with five isoprenoid units and appears in the overview of *Fusarium* mycotoxins in 1993 (Randazzo et al., 1993). The chemical structure of FP has been determined by NMR and mass spectrometry, and the configurations of the four chiral carbons have been established by distance geometry and X-ray techniques (Mettere Santini et al., 1996). Its biosynthetic pathway is consistent with that of retigeranic acid (Manetti et al., 1995; Santini et al., 1996). FP is biologically active toward *Artemia salina* L. larvae, SF-9 insect cells, IARC/LCL 171 human B lymphocytes (Logrieco et al., 1996) as well as teratogenic to chicken embryos (Ritieni et al., 1997a). The occurrence of FP and BEA together with fumonisin B_1 has been largely reported so far (Krska et al., 1997; Ritieni et al., 1997b). This paper describes the optimization of a relatively simple and sensitive method for monitoring and quantifying these mycotoxins in one single HPLC (high performance liquid chromatography) run.

EXPERIMENTAL PROCEDURES

Water for the HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA). Organic solvents (HPLC grade) were purchased from Carlo Erba (I). The standards of BEA and ENN mixture were purchased from Sigma Chemical Co. (St. Louis, MO). The ENNs mixture contained the following percentages: ENN-B_1 54%, ENN-B 19%, ENN-A_1 20%, and ENN-A 3%.

A stock of FP was obtained from *Fusarium proliferatum* ITEM 1494 as previously described (Ritieni et al., 1995) and

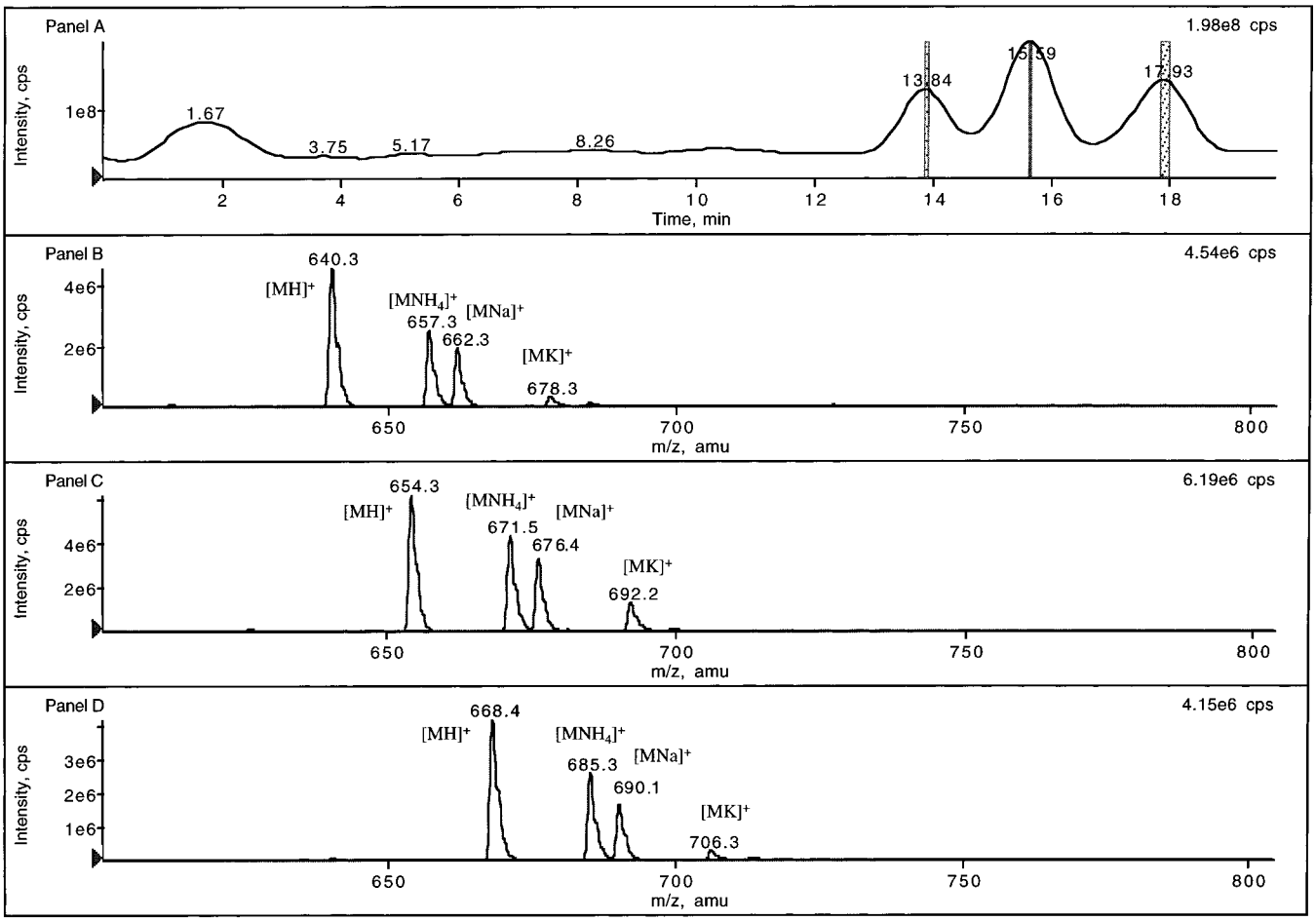

Figure 3. Intensity current (cps) of three separated peaks eluted at 13.93 min, 15.68, and 18.01 min (panel A) and mass spectra of the respective peaks showing a molecular ion of 640.4 *m/z* (panel B), 654.4 *m/z* (panel C), and 668.4 *m/z* (panel D). These molecular masses correspond to ENN B, B_1, and A_1. respectively. Adducts with ammonium, sodium, and potassium ions are also evident.

Table 1. HPLC Linearity, Detection Limit (ng), Coefficient of Regression, and Recovery (%) for Each of the Tested Compound

mycotoxin	HPLC linearity (μg/mL)	detection limit (ng)	coefficient of regression	mean recovery from water (%) and variance	range of recovery (%)
ENN-A_1	2.1−127	1.3	0.988	58.6 (4.20)	56.10−58.60
ENN-B_1	5.6−338	3.6	0.990	62.5 (3.82)	60.80−63.20
ENN-B	2.2−135	1.2	0.989	66.5 (4.88)	64.20−68.70
BEA	1−500	20	0.997	74.6 (4.85)	72.10−76.10
FP	1−500	0.5	0.998	56.1 (3.29)	54.00−56.80

its purity was confirmed by GC-MS analysis as reported by Ritieni et al., (1997b).

Preparation of Samples. Calibration curves of mycotoxins were performed using a standard mixture formed by the addition of ENN mixture (0.9 mg/mL), BEA (2.5 mg/mL) and FP (3.5 mg/mL) and kept as a methanol stock solution. Spiking experiments and related recovery were performed by adding different amounts of the mycotoxins mixture. In particular, ENN mixture (560 μg), BEA (140 μg), and FP (103 μg) were added to 10 mL of distilled water. All of the artificially contaminated solutions were then extracted three times with 10 mL portions of chloroform, for three times, and the combined organic phases were dried over sodium sulfate, evaporated under reduced pressure, and resuspended in 200 μL of methanol before HPLC analysis.

HPLC analyses were performed using LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Shiseido Capcell Pak C_{18} (250 × 4.6 mm, 5 μm) column was used. HPLC conditions were set up using a constant flow at 1.5 mL/min and CH_3CN-H_2O (65:35 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 70% acetonitrile in 10 min. After 1 min at 70% CH_3CN, the mobile phase was taken back to the starting conditions in four minutes. FP was detected at 261 nm, BEA and ENN at 205 nm.

All samples were filtered through a 0.22 μm syringe filter (Millipore, Yonezawa, Japan) prior to injection (20 μl) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified samples to pure standards. A further confirm action was performed by co-injecting pure standards together with each sample. Mycotoxin quantification was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

LC-MS. HPLC conditions described above for the analytical separations were applied, but a different HPLC system was used. A Perkin-Elmer LC series 200 connected to a 785A UV/VIS detector was coupled with an API-100 single quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada). A flow rate of 20 μL min^{-1} was split from the LC eluent into the ion spray source. A probe voltage of 5300 V and a declustering potential of 50 V were used. Full-scan spectra were acquired from 400 to 800 amu using a step size of 0.5 amu and a dwell time of 4.2 ms. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adducts of polypropylene glycol.

RESULTS AND DISCUSSION

The calculated recovery of each mycotoxin was: BEA 74.6%, FP 56.1%, ENN-B 66.5%, ENN-B_1 62.5%, and 58.6% for ENN-A_1. Although chloroform did not give the best recovery (Ritieni et al., 1998), this choice was balanced by the advantage of a fast separation of the organic phase from the polar one, thus allowing a rapid concentration of samples without thermal stress. Retention times recorded for authentic standards were FP 5.94 min, BEA 8.64 min, ENN-B 9.81 min, ENN-B_1 10.43, and 11.26 min for ENN-A_1 (Figure 2).

HPLC detection limits of mycotoxins were calculated by injection of different concentrations of each. The lowest detectable amounts injected were of 0.5 ng for FP, 20 ng for BEA, and 3.6 ng for ENN-B_1 ($S/N = 4$),

Table 2. Calibration Curve Responses for Each of the Tested Compounds

compds	concd injected (μg/mL) {value a}	response (μg/mL) {value b}	ratio response concd injected {a/b}	ratio (%) ((a/b)/mean) × 100
ENN-A1				
1	2.10	1.85	0.88	91.77
2	12.70	11.97	0.94	98.18
3	63.00	64.50	1.02	106.65
4	127.00	126.05	0.99	103.39
mean			0.96	
ENN-B1				
1	5.60	4.85	0.87	90.24
2	33.80	32.20	0.95	99.26
3	169.20	173.70	1.03	106.96
4	338.00	335.90	0.99	103.54
mean			0.96	
ENN-B				
1	2.20	1.91	0.87	90.70
2	13.50	12.65	0.94	97.89
3	67.50	69.65	1.03	107.80
4	135.00	133.90	0.99	103.62
mean			0.96	
BEA				
1	1.00	1.12	1.12	109.92
2	10.00	9.62	0.96	94.41
3	100.00	100.03	1.00	98.17
4	500.00	496.78	0.99	97.51
mean			1.02	
FP				
1	1.00	1.10	1.10	109.75
2	10.00	9.33	0.93	93.09
3	100.00	97.47	0.97	97.25
4	500.00	500.70	1.00	99.91
mean			1.00	

which was the main component of ENNs mixture. Precision of measurements was readily determined by triplicate injections performed under identical conditions and found to have a RSD (relative standard deviation) of 2.12%. Recovery experiments were carried out in duplicate with three different spiking levels for each mycotoxin; results are presented in Table 1 where the analytical linearity, detection limit, coefficient of regression, and recovery for each of the tested compounds are reported.

HPLC calibration curve showed a linear correlation between sample concentration and peak area. It was performed twice on two different days and each concentration point was injected at least in duplicate. The calculation of a four-point calibration curve was based on linear regression followed by the least-squares method. The single response/mass ratio of each calibration point was calculated and did not differ more than ±10% from the mean of the response mass/ratio of all points of curve. This procedure was done according to van Trijp and A. H. Roos (van Trijp and Roos, 1991); results are shown in Table 2. In Figure 2 a complete HPLC profile obtained with the standard solution of mycotoxins is illustrated. It is worth noting the complete chromatographic separation of the five mycotoxins, including the different ENN compounds. To ensure the structural identification of ENNs, LC-MS analyses were

carried out (Figure 3). The intensity (cps) of three separated peaks eluted at 13.93, 15.68, and 18.01 min is shown in Figure 3 (panel A). In Panels B, C, and D the mass spectra of the respective peaks having a molecular ion of 640.4 *m/z*, 654.4 *m/z*, and 668.4 *m/z* are reported. Adducts with ammonium, sodium, and potassium ions are also evident. These molecular ions are consistent with the molecular weight of ENN-B, ENN-B_1 and ENN-A_1, respectively. The mass spectra confirmed that ENN-B is the first metabolite eluted, immediately followed by ENN-B_1 and ENN-A_1. The retention times recorded agreed with the increased lipophilicity of the lateral chain of ENN-B to ENN-A_1 (Figure 1b). Nuclear magnetic resonance (NMR) spectra of the three ENN peaks collected separately by HPLC were consistent with data reported in the literature (Visconti et al., 1992).

In conclusion, the chromatographic method described above permits one to obtain in a single run of 15 min, a complete separation and quantification of five different mycotoxins with good sensitivity and reproducibility. Future work is needed to apply this procedure to analyze foodstuffs and animal feeds for the presence of these mycotoxins.

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LITERATURE CITED

Arai, M.; Hibino, T.; Tumorigenity of citrinin in male F344 rats. *Cancer Lett*. **1983**, *17*, 281−287.

Busby, W. F., Jr.; Wogan, G. N. Aflatoxins. In *Chemical carcinogens*; Searle, C. E., Ed.; American Chemical Society: Washington, DC, 1984; pp 945−1136.

Grove, J. F.; Pople, M. The insectidial activity of beauvericin and enniatin complex. *Mycopathologia* **1980**, *70*, 103−105.

Gupta, S.; Krasnoff, S. B.; Underwood, N. L.; Renwick, J. A. A.; Roberts, D. W. Isolation of beauvericin as an insect toxin from *Fusairum semitectum* and *Fusarium moniliforme* var. *subglutinans. Mycopathologia* **1991**, *115*, 185−189.

Kleinkauf, H. Antibiotics polypeptides-biosynthesis on multifunctional protein templates. *Planta Med*. **1979**, *35*, 1−18.

Kostecki, M.; Szczesna, J.; Chelkowski, J; Wisniewska, H. Beauvericin and moniliformin production by Polish isolates of *Fusarium subglutinans* and natural co-occurrence of both mycotoxins in maize samples. *Microb. Alim. Nutr*. **1995**, *13*, 67−70.

Krska, R.; Lemmens, M.; Schuhmacher, R.; Grasserbauer, M.; Pronczuk, M.; Wisniewska, H.; Chelkowski, J. Accumulation of the mycotoxin beauvericin in kernels of corn hybrids inoculated with Fusarium subglutinans. *J. Agric. Food Chem*. **1997**, *44*, 33665-3667.

Logrieco, A.; Moretti, A.; Fornelli, F.; Fogliano, V.; Ritieni, A.; Caiaffa,M. F; Randazzo, G.; Bottalico, A.; Macchia, L. Fusaproliferin Production by *Fusarium subglutinans* and Its Toxicity to *Artemia salina*, SF-9 Insect Cells, and IARC/LCL 171 Human B Lymphocytes. *Appl. Environ. Microbiol*. **1996**, *62*, 3378−3384.

Logrieco, A.; Moretti, A.; Ritieni, A.; Chelkowski, J.; Altomare, C.; Bottalico, A.; Randazzo, G. Natural occurrence of beauvericin in preharvest *Fusarium subglutinans* infected corn ears in Poland. *J. Agric. Food Chem*. **1991**, *41*, 2149−2152.

Macchia, L.; Di Paola, R.; Fornelli, F.; Nenna, S.; Moretti, A.; Napoletano, R.; Logrieco, A.; Caiaffa, M. F.; Tursi, A.; Bottalico, A. *Abstracts International on Fusarium*, Martina Franca, Italy, May 9−13, 1995; p *72*.

Manetti, C.; Fogliano, V.; Ritieni, A.; Santini, A.; Randazzo, G.; Logrieco, A.; Mannina, L.; Segre, A. L. Determination of the structure of Fusaprolferin by ^{1}H NMR and distance geometry. *Struct. Chem*. **1995**, *6*, 183−189.

Moretti, A.; Logrieco, A.; Bottalico, A.; Ritieni, A.; Randazzo, G. Production of beauvericin by *Fusarium proliferatum* from maize in Italy. *Mycotoxin Res*. **1994**, *10*, 73−78.

Ovchinnikov, Yu. A.; Ivanov, V. T.; Evstratov, A. V.; Mikhaleva, I. I.; Bystrov, V. F.; Portnova, S. L.; Balashova, T. A.; Meshcheryakova, E. N.; Tulchinsky, V. M. The enniatin ionophores. Conformation and ion binding properties. *J. Pept. Protein Res*. **1974**, *6*, 465−498.

Peeters, H.; Zocher, R.; Kleinkauf, H.; Synthesis of beauvericin by a multifunctional enzyme *J. Antibiot*. **1988**, *41*, 352−359.

Pocsfalvi, G.; Di Landa, G.; Ferranti, P.; Ritieni, A.; Randazzo, G.; Malorni, A. Observation of Noncovalent Interactions Between Beauvericin and Oligonucleotides Using Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom*. **1997**, *11*, 265−272.

Prince, R. C.; Crofts, A. R.; Steinrauf, L. K. A comparison of beauvericin, enniatin and valinomycin as calcium transporting agents in liposomes and chromatophores *Biochem. Biophys. Res. Commun*. **1974**, *54*, 697−703.

Randazzo, G.; Fogliano, V.; Ritieni, A.; Mannina, L.; Rossi, E.; Scarallo, A.; Segre, A. L. Proliferin, a new sesterterpene from *Fusarium proliferatum*. *Tetrahedron* **1993**, *49*, 10883−10896.

Ritieni, A.; Fogliano, V.; Randazzo, G.; Scarallo, A.; Logrieco, A.; Moretti, A.; Mannina, L.; Bottalico, A. Isolation and characterization of Fusaproliferin, a new toxic metabolite from *Fusarium proliferatum*. *Nat. Toxins* **1995**, *3*, 17−20.

Ritieni, A.; Monti, S. M.; Randazzo, G.; Logrieco, A.; Moretti, A.; Peluso, G.; Ferracane, R.; Fogliano, V. Teratogenic Effects of Fusaproliferin on chicken embryos. *J. Agric. Food Chem*. **1997a**, *45*, 3039−3043.

Ritieni, A.; Moretti, A.; Logrieco, A.; Bottalico, A.; Randazzo,G.; Monti, S. M.; Ferracane, R.; Fogliano, V. Occurrence of Fusaproliferin, Fumonisin FB_1 and Beauvericin in maize from Italy. *J. Agric. Food Chem*. **1997b**, *45*, 4011−4016.

Ritieni, A.; Monti, S. M.; Randazzo, G.; Moretti, A.; Ferracane, R.; Fogliano, V. Stability of Fusaproliferin, a mycotoxin from *Fusarium J. Sci. Food Agric*. **1998**, in press.

Santini, A.; Ritieni, A.; Fogliano, V.; Randazzo, G.; Mannina, L.; Logrieco, A.; Benedetti, E. Structure and Absolute Stereochemistry of Fusaproliferin, a Toxic Metabolite from *Fusarium proliferatum*. *J. Nat. Prod*. **1996**, *59*, 109−112.

Schlatter, C. H., Studer-Rohr, J.; Rasonyi, T. H. Carcinogenicity and kinetic aspects of ochratoxin A. *Food Addit. Contam*. **1996**, *13*, 43−44.

Strongman, D. B.; Stranz, G. M.; Giguere, F.; Yu, C. M.; Calhoun, L.; Enniatins from *Fusarium avenaceum* budworm larvae, *Choristoneurea fumiferana* (Clem.). *J. Chem. Ecol*. **1988**, *14*, 753−764.

van Trijp, J. M. P.; Roos, A. H.; Model for the calculation of calibration curves RIKILT-DLO Report 91.02, January 1991.

Visconti, A.; Blais, L. A.; ApSimon, J. W.; Greenhalgh, R.; Miller, D. Production of Enniatins by *Fusarium acuminatum* and *Fusarium compactum* in Liquid Culture: Isolation and Characterization of Three New Enniatins, B_2, B_3, and B_4. *J. Agric. Food Chem*. **1992**, *40*, 1976−1982.

Zocher, R.; Keller, U.; Kleinkauf, H. Enniatin synthetase, a novel type of multifunctional enzyme catalyzing depsipeptide synthesis in *Fusarium oxysporum*. *Biochemistry* **1982**, *21*, 43−48.

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