

Determination of *Fusarium* Mycotoxin Beauvericin in *Bombyx batryticatus* by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Fusarium Mycotoxin beauvericin has been proven to be a potent antibiotic compound useful for bio-control and a potential antifungal and anticancer agent for human. The aim of this study was to determine the level of *Fusarium* mycotoxin beauvericin in *Bombyx batryticatus* using LC-MS/MS and observe its content changes in production process within 12 days. The method yielded satisfactory precision, stability and repeatability (RSD: 0.63 %, 0.96 %, 1.16 %). Average recovery of three spiked levels was 100.3 % with RSD 4.54 %. The level of beauvericin changes with a certain law in dead silkworm bodies. *Fusarium* mycotoxin beauvericin content was almost zero in the first four days, then extended increasing and reached its maximum in 10th day, which was high up to 43.08 µg/g. The trend of *Fusarium* mycotoxin beauvericin level declined from 10th day. This is the first study to report the content of *Fusarium* mycotoxin beauvericin in *B. batryticatus*.

Key Words: *Bombyx batryticatus*, *Fusarium* Mycotoxin beauvericin, LC-MS/MS.

INTRODUCTION

Mycotoxins are secondary metabolites produced by different genera of filamentous fungi¹, mostly *Aspergillus*, *Fusarium* and *Penicillium*². *Fusarium* mycotoxin beauvericin is a small family of mycotoxins which is a cyclic hexadepsipeptide consisting of three alternating *N*-methyl-L-amino acid and D- α -hydroxyisovaleric acids (Fig. 1). *Fusarium* mycotoxin beauvericin was firstly isolated from a culture of the insect-pathogenic fungus *Beauveria bassiana*³, which was recognized as the causative agent for heavy losses of the sericulture. Beauvericin shows antibacterial^{4,5}, antifungal, insecticidal activities⁶, even significant cytotoxic activity to various human cancer cell lines^{7,8}. Therefore, *Fusarium* mycotoxin beauvericin can be applied as a potential antibiotic and anticancer agent for human.

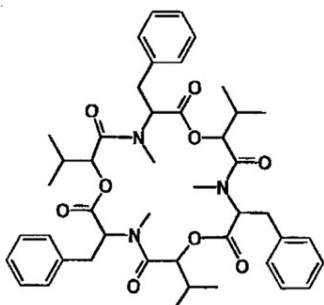


Fig. 1. Chemical structure of *Fusarium* mycotoxin beauvericin

B. batryticatus or *Bombycis corpus*, a batryticated and white-stiff silkworm, is a drug consisting of the dried larva of silkworm, *Bombyx mori* L, dead and stiffened due to the infection of *Beauveria bassiana*⁹. *B. batryticatus* has been always used in chinese traditional medicine to treat diseases such as palsy, headache, convulsion, stroke induced speech problem and tremor.

As *B. bassiana* can produce beauvericin, we speculate that *B. batryticatus* infected by the fungus contain a certain amount of beauvericin and its content changes in a regular way as time goes on. A number of methods have been developed to detect beauvericin in various matrices, however, is mainly focus on grain^{10,11} or food matrices^{12,13} and animal feed^{2,14}. The preparation methods are broadly similar including sample extraction of the analytes using different solvents and removal of impurities using different types of PSE columns. Up to date, we firstly determined beauvericin content in *B. batryticatus* in the present study. Besides, we presume that the content of beauvericin vary along with the growth of *B. bassiana* in silkworm and appear a definite rule. So we also investigated its content changes everyday within 12 days. There is almost no report on production of beauvericin besides *Fusarium. redolens* Dzf2 mycelial culture¹⁵. Furthermore, this may supply another potential choice of large-scale production of beauvericin with low-cost and simple process.

EXPERIMENTAL

Preparation of *B. batryticatus* samples: *B. batryticatus* samples were made in May 2011. The silkworm larvae of strain (Qiufeng × Baiyu) were reared in mass on mulberry leaves up to the fourth moult. Conidia of *B. bassiana* were collected from the confluent white fungal mat over the entire larval body and an inoculum was made up to contain approx. 1×10^7 conidia/mL¹⁶. The inoculation was done by surface-application on to healthy larvae just after resumption of the fifth instar and the silkworms were reared continuously under more favourable circumstances (25 °C, humidity 90 %). The dead mummified larvae were collected and kept till they were covered with white conidiophores. Samples were collected at 24 h intervals. *B. batryticatus* were frozen-dried by Christpha 2-4 freeze drier (Bmh Instruments Co. Ltd.) prior to use.

Beauvericin standard was purchased from Sigma (St. Louis, MO, USA). Stock solution of 1 µg/µL of beauvericin were prepared in acetonitrile and stored at -20 °C. Acetonitrile (Tedia, USA) was of LC quality. The water used was Milli-Q water purified with a Millipore Milli-Q Plus System. Silica Solid Phase Extraction columns (ProElut, 100 mg, 1 mL) were purchased from Dikma (Beijing, China).

Sample preparation: *B. batryticatus* samples were ground with a laboratory mill. One gram sample was homogenized with 20 mL of 84 % acetonitrile in water^{11,17} for 3 min and thereafter extracted for 0.5 h in an ultrasonic bath (500 W) for 2 times. The extracted samples were centrifuged at 8000 rpm for 5 min. Crude extracts were put together and homogenized. 1 mL crude extract was cleaned with SPE using a silica column^{5,12,18}. Before loading the sample onto the column, the column was conditioned with 2 mL acetonitrile. A sample was passed through the conditioned column and the eluate was collected into a test tube. The column was further washed with 5 mL of acetonitrile, which was also collected. Eluates were pooled and concentrated under a stream of nitrogen, redissolved into 1 mL 50 % acetonitrile in water and mixed thoroughly with a Vortex-Genie 2 test tube mixer. The dissolved sample was filtered through a 0.2 µm syringe filter (Millipore). The samples were diluted to 100 times before injecting into a liquid chromatography-tandem mass spectrometer (LC-MS/MS).

A waters acquity ultra-performance liquid chromatography (UPLC) system coupled to a Quattro Ultima Pt Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) probe was used to analyze the samples. The analytical column was a BEH C₁₈, 1.7 µm, 2.1 × 100 mm with inline filter, 0.22 µm (Waters).

HPLC conditions: The column was kept at 40 °C. The injection volume was 10 µL. The mobile phase consisted of variable mixtures of mobile phase A (acetonitrile) and mobile phase B (10 mM ammonium acetate) at a flow rate of 0.3 mL/min with a gradient elution program. The gradient elution started at 80 % mobile phase B with a linear increase to 100 % in 3 min. This state kept for 0.5 min before mobile phase B was decreased to 80 % during the next 0.1 min and then held for another 3 min.

MS/MS detection: The mass spectrometer was operated in the positive electrospray ionization (ESI⁺) mode. The parameters of the MS were optimized using the standard solutions and the best response was recorded with the following parameters: cone voltage 45 V, capillary voltage 3.80 kV, source temperature 150 °C, desolvation temperature 350 °C and flow rate of desolvation 500 L/h. Nitrogen and argon were used as spray and collision gas, respectively. MRM-technique was used for identification and quantification, in which protonated molecule [M+H]⁺ of the analyte beauvericin (*m/z* 785) was fragmented in the collision cell to the product ions: *m/z* 243.9 and 261.9. Product-ion *m/z* 243.9 was used for the quantification of beauvericin. However, the quantification of beauvericin was achieved by comparing the peak areas of the samples with the corresponding calibration plot of the beauvericin standard (Fig. 2).

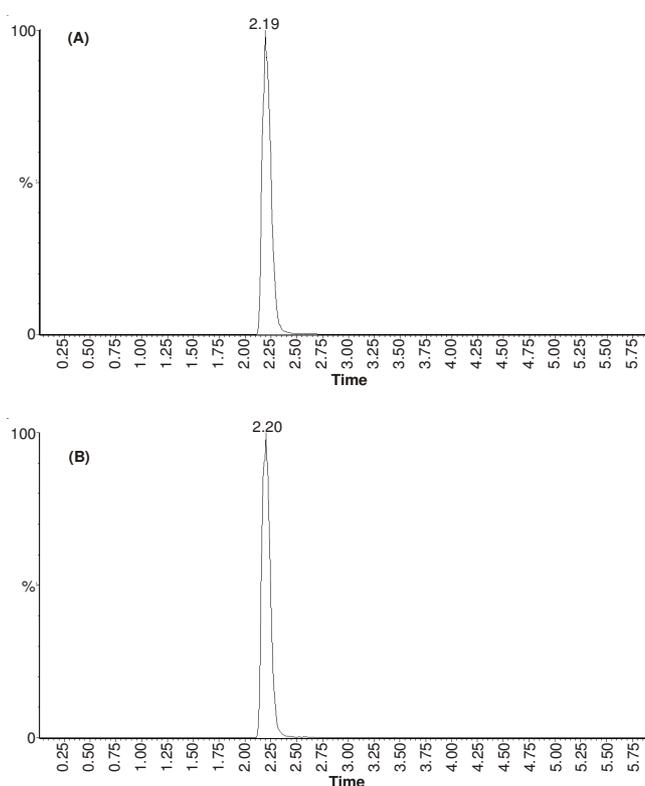


Fig. 2. HPLC chromatogram of beauvericin standard (A), *B. batryticatus* samples (B)

Method validation: Five validation parameters were determined to describe the performance of the method: Linearity, precision, stability, recovery and repeatability, limit of detection (LOD) and limit of quantification (LOQ). Signal-to-noise (S/N) ratios of 3 or above were considered acceptable for LOD and S/N of 10 or above for LOQ. Replicates ($n = 3$) of spiked samples (the beauvericin content had already known in every matrix sample), at three concentration levels and calibration curve were analyzed.

RESULTS AND DISCUSSION

Purification and qualitative identification of beauvericin in *B. batryticatus*: 84 % acetonitrile in water, which is reported to receive the best recovery of beauvericin, was

chosen to be the extraction solvent. Utilization of silica SPE overcomes shortcomings of loss-causing liquid-liquid extraction. Beauvericin does not have any chemical group that is easily accessible for derivatization, but it is able to form complexes with different metal ions such as Na^+ , K^+ , Ca^+ and NH_4^+ , which could be fragmented in various ways and lead to problems in quantification. By using ammonium acetate in the mobile phase, we could exclude all other complexes for beauvericin, except $\text{M}+\text{NH}_4$. In MS trace analysis, MRM mode was used, which means two specific ions produced by the analyte were detected.

Under the same HPLC-MS/MS condition, *B. batryticatus* was analyzed and the chromatogram results showed that one single peak was eluted with the same retention time of 2.20 min to standard solution. Besides, when spiked by beauvericin standard, chromatographic peak area increased linearly (Fig. 2). This shows that *B. batryticatus* contains beauvericin and beauvericin can be well separated by HPLC and analyzed qualitatively and quantitatively according to its retention time and peak area. Finally, the mean concentration of beauvericin was 21.64 $\mu\text{g/g}$.

Method validation: Using the concentration of beauvericin standard solution as abscissa and the peak area as response, a better linear chart at the concentration of 5-1000 ng/mL was constructed: $y = 8270.03x + 11519.5$ ($r^2 = 0.9973$). Apparatus precision was evaluated by determination of *B. batryticatus* for five successive times and solution stability was by the analysis of a solution of the sample every 2 h. Spiked solutions were prepared in acetonitrile and appropriate amounts added to *B. batryticatus* samples. Repeatability of the method was evaluated by the analysis of three parallel *B. batryticatus* samples and recovery was by analyzing spiked samples at three concentration levels. The determined LOD and LOQ were calculated according to the guidelines of the German norm DIN 3264 which are derived from the y-intercept and its confidence interval after linear regression, which is 4 $\mu\text{g/g}$ and 12 $\mu\text{g/g}$, respectively. In general, the method yielded satisfactory precision, stability and repeatability, whose RSDs were 0.63, 0.96 and 1.16 %. Also a better mean recovery and precision for beauvericin was obtained from spiked *B. batryticatus* samples (Table-1).

Spiking level	Level 1 (10 $\mu\text{g/g}$)	Level 2 (30 $\mu\text{g/g}$)	Level 3 (60 $\mu\text{g/g}$)
Recovery (%)	104.3 \pm 3.15	100.9 \pm 3.12	95.7 \pm 2.70
Average recovery (%)	100.3 \pm 4.54		

Changes of beauvericin content in silkworm bodies post-inoculation by *B. bassiana*: The level of beauvericin in dead silkworm bodies changes with a certain law from 1st to 12th day after inoculation presented in Fig. 3. In the previous 4 days beauvericin's level was close to zero, then increase steadily until it reached the highest level in day 10, at which point it dipped sharply in the next 2 days. The level of *B. batryticatus* sample was 21.64 $\mu\text{g/g}$, which was between that of day 7 and day 8. The highest content of day 10 reached 43.08 $\mu\text{g/g}$ more than twice that of *B. batryticatus* sample. The next 2 days'

content was almost as high as that of day 7. Since the inoculated silkworms began to deteriorate from 9th day, we speculated that migration downwards of beauvericin level in 11th day was because the life-cycle of the fungus was completed.

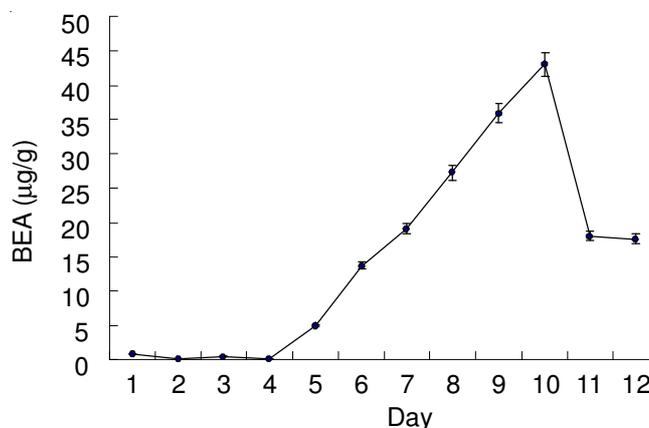


Fig. 3. BEA content in silkworm bodies inoculated with *B. bassiana* from fifth moult

Conclusion

Methods for the quantitative determination of mycotoxins in animal feed², grains¹⁰ or eggs¹² were already available. Based on those recommendations, this study has successfully shown the potential of LC-MS/MS method to rapidly determine beauvericin's level in *B. batryticatus*. Compared to those published papers that detect beauvericin contamination in food matrices, the values of LOD and LOQ were higher. This is probably because of the sensitivity of LC-MS/MS to beauvericin in the fungus-insect complex. Also, the ingredients of the complex were too complicated so that matrix effect was a little stronger, which led to the higher LOD and LOQ.

The validation results showed that the sample preparation method developed for detecting beauvericin in food matrices was well applicable for the determination in *B. batryticatus*. The results showed *B. batryticatus* sample contained sufficient beauvericin which was as high as 21.64 $\mu\text{g/g}$. Four days before the whole preparation of *B. batryticatus* beauvericin's level was almost close to zero and then beauvericin increased linearly with the passage of time before the 10th day. After that beauvericin's level declined rapidly in 11th day and that of day 12 remained almost the same. So we figure that the change law of beauvericin's level supports the growth rule of *Beauverina bassiana* within silkworm body as reported¹⁶. Kumar *et al.*¹⁶ confirmed that the life-cycle of *Beauverina bassiana* on silkworm was completed in 7-8 days. However beauvericin's level reached the highest in day 10. Time difference between the paper and our practice may be because of some reasons that lag the whole life-cycle including differences in infectious ability of the fungus conidia, varieties of silkworm, manual operation of inoculation and culture conditions.

Thus *B. batryticatus* samples contain sufficient beauvericin and we already know 10th day's level is the highest. This demonstrates the feasibility and promising potential of preparation of *B. batryticatus* for efficient and large-scale production of beauvericin and related bioactive compounds.

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