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# A Validated RP-HPLC Method for the Determination of Citrinin in Xuezhikang Capsule and other *Monascus*-Fermented Products

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**Abstract:** Citrinin is a toxic product usually produced during the *Monascus* fermentation. The presence of citrinin in xuezhikang capsule has been a concern due to its ingredient which is derived from *monascus*-fermented rice. A rapid and sensitive RP-HPLC method with fluorescence detection at $\lambda_{ex}$ = 331 nm and $\lambda_{em}$ = 500 nm for analysis of citrinin in *Monascus*-fermented products was developed to analyze citrinin in *Monascus*-fermented products. The chromatography was performed with mobile phase containing acidified water and acetonitrile. The calibration curve was linear (r = 0.9999) over a range of 0.0107- 0.537 µg/mL. The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.187 ng/mL and 0.6 ng/mL respectively. The analysis of xuezhikang capsules using the developed method suggested that the product does not contain detectable citrinin and the result has been further confirmed using independent LC-MS/MS analysis. The proposed method has also been applied to analyze 11 samples of other *Monascus*-fermented products. The results suggested that there were no detectable citrinin in 4 of the 11 samples, however citrinin with the levels between 0.10-594 ng/kg has been detected in the other 7 samples. It indicates that the proposed method can also be applied to carry out the quantitative detection of citrinin for other *Monascus*-fermented products.

**Keywords:** HPLC, LC-MS/MS, Citrinin, Xuezhikang capsule, *Monascus*

## Introduction

Cintrinin, a toxic product of secondary metabolite of fungi, such as *Aspergillus*, *Penicillium*, and *Monascus* species, is known to lead to serious health problems[1]. Generally, citrinin is

present in a trace concentration (0.1-500 mg/kg), compared with other *Monascus* metabolites. Citrinin [$C_{13}H_{14}O_5$, IUPAC: (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid; CAS No.: 518-75-2] (Figure 1), is an acidic lemon-yellow crystal with maximal UV absorption at 250 nm and 333 nm (in methanol), melting at 172 °C. The reversed-phase HPLC with sensitive fluorescence detection is the most effective method for the determination of citrinin[2-4]. According to the Chinese national standard method for the determination of citrinin in *Monascus*-fermented products, an ultrasonic-extraction with toluene-ethyl acetate-formic acid = 7:3:1 is used for the pretreatment of the *Monascus*-fermented products[5].

In this work, we developed a selective and sensitive PR-HPLC method that can conveniently detect the lowest possible amount of 0.187 ng/mL citrinin, which is more sensitive than the recent report of 0.5 ng/mL detection limit[4]. Further, how much citrinin is lost in the pretreatment process was investigated by recovery studies.

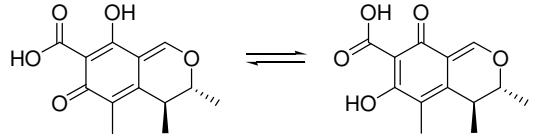



**Figure 1.** Chemical structures of citrinin isomers

## Experimental

Citrinin was purchased from Sigma (Shanghai, China). Xuezhikang capsule (Xuezhikang) was produced by Beijing WBL Peking University Biotechnology Co., Ltd., China. The commercially available *Monascus*-fermented products were obtained from the local market. Acetonitrile and methanol were of HPLC grade and all other reagents were analytical grade.

### *Instrumentation*

The chromatographic system consisted of Shimadzu LC-20A separations module and a Shimadzu RF-20A Fluorescence Detector. Fluorescence detection was performed with the Shimadzu RF-20A fluorescence detector with $\lambda_{ex}$ = 331 nm and $\lambda_{em}$ = 500 nm. A column of Aichrombond-AQ $C_{18}$ (250 mm × 4.6 mm i.d., 5 μm) was used and the column temperature was set at 25 °C. An Agilent 1200 HPLC separation system was used to deliver samples into a Bruker micrOTOF-QII mass spectrometer.

### *Preparation of standard solution*

The standard stock solution was prepared by dissolving accurately weighed 5.37 mg of citrinin standard in 100 mL of methanol (final concentration, 53.7 μg/mL). Different calibration standards ranging from 0.01074, 0.0537, 0.1074, 0.2685 and 0.537 μg/mL were prepared by appropriate dilution of standard stock solution with methanol.

### *Preparation of sample solution*

An assay sample (Xuezhikang or other *Monascus*-fermented products) was milled and passed through 80-mesh sieve. Accurately weighed 1.5 g of sample powder was transferred to a 15 mL PTEE centrifuge tube, and ultrasonic-extracted with 10 mL toluene-ethyl acetate-formic acid = 7:3:1 for 20 min, 3 times. The combined extract solution was centrifuged to remove undissolved residue and the supernatant was evaporated under vacuum to afford citrinin-enrichment crude extract. The crude extract was dissolved with 10 mL methanol and filtered through a 0.45 μm membrane filter for detection.

*Chromatographic conditions*

The mobile phase A was acetonitrile and mobile phase B was acidified water (pH 2.5 adjusted with phosphoric acid). The gradient elution program has been developed and optimized for better, accurate and consistent results. The gradient eluting system was shown in Table 1. The injection volume was 20 µL and the flow rate was 1.0 mL/min.

**Table 1.** Gradient elution system in HPLC

| Time, min | A, % | B, % |
|---|---|---|
| 0 | 53.5 | 46.5 |
| 14 | 53.5 | 46.5 |
| 15 | 100 | 0 |
| 22 | 100 | 0 |
| 23 | 53.5 | 46.5 |
| 30 | 53.5 | 46.5 |

## Results and Discussion

The applied chromatographic conditions permitted a good resolution of citrinin in standard solution (A) and in sample solution (B) (Figure 2). The LC method was validated for the parameters reported below.

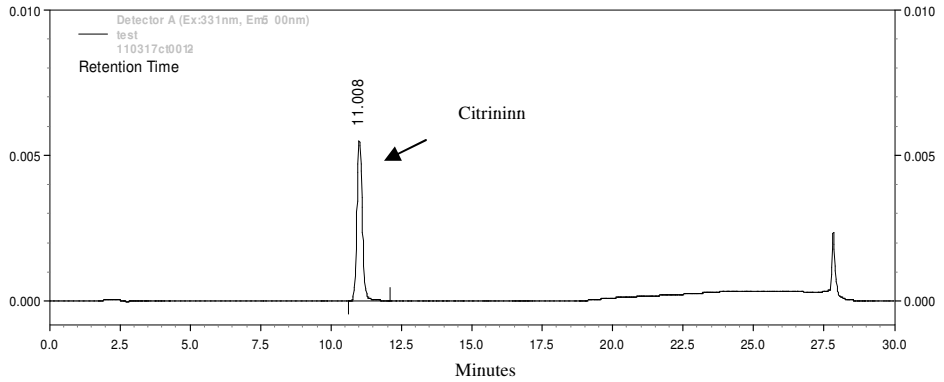



**Figure 2(A).** Typical chromatogram of citrinin

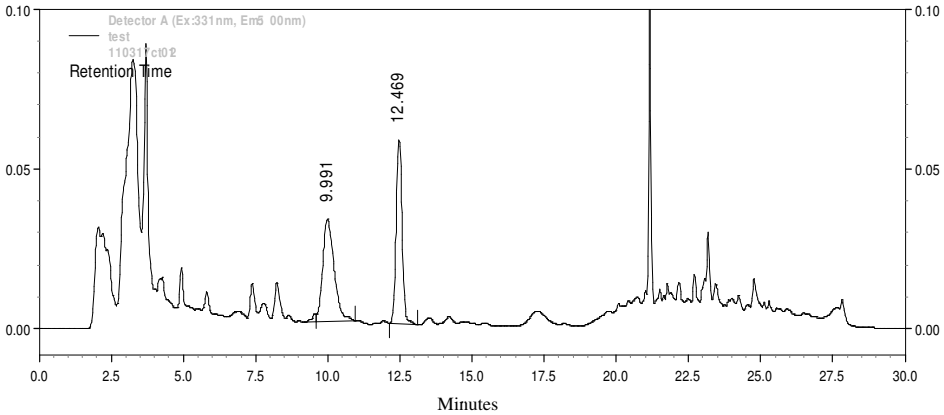



**Figure 2(B).** Typical chromatogram of Xuezhikang

*Linearity*

The calibration standards were chromatographed using the mobile phase, and the linearity of peak areas *versus* corresponding concentrations was studied from 0.01074 - 0.537 µg/mL for citrinin. A linear response was observed over the examined concentration range. The results are tabulated in Table 2.

**Table 2.** Results of the data analysis for the linearity of citrinin

| Parameters | HPLC |
|---|---|
| Concentration range, µg/mL | 0.01074 - 0.537 |
| Regression equation | Y = 34335.93X - 1363.90 |
| Correlation coefficient (r) | 0.9999 |

*Limit of detection and limit of quantitation*

The limit of quantitation (LOQ) was the lowest concentration of the sample assayed when the signal/noise ratio was at least 10:1. The limit of detection (LOD) was defined as a signal/noise ratio of 3:1. The LOQ and LOD were found to be 0.6 ng/mL and 0.187 ng/mL respectively.

*Precision and recovery*

The precision of the method was evaluated by analyzing the standard solution of 0.1074 µg/mL citrinin with five replicates. The results are shown in Table 3.

**Table 3.** The result of precision assay

| No. | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Peak area of citrinin | 71482 | 71218 | 71413 | 71413 | 71479 |
| RSD, % | | | 0.15 | | |

The recovery tests were conducted to evaluate the extraction method aforementioned. They were performed by adding known amounts of standard stock solutions to the xuezhikang samples and preparing solutions according to the preparation of sample solution. The percentage of recovery was calculated by comparing the determined amount of citrinin standard with the added amount. The calculation of % recovery is tabulated in Table 4.

**Table 4.** The result of recovery analysis

| No. | Weight of Xuezhikang, g | Citrinin in Xuezhikang, µg | Spiked Citrinin, µg | Calculated Citrinin, µg | Recovery, % | Average Recovery, % | RSD, % |
|---|---|---|---|---|---|---|---|
| 1 | 1.5029 | undetected | 5.37 | 5.15 | 95.90 | 96.68 | 0.64 |
| 2 | 1.5037 | undetected | 5.37 | 5.18 | 96.46 | | |
| 3 | 1.5031 | undetected | 5.37 | 5.22 | 97.21 | | |
| 4 | 1.5050 | undetected | 5.37 | 5.19 | 96.65 | | |
| 5 | 1.5009 | undetected | 5.37 | 5.24 | 97.60 | | |
| 6 | 1.5068 | undetected | 5.37 | 5.17 | 96.28 | | |

Natural occurrence of citrinin in Xuezhikang and other *Monascus*-fermented products (including red yeast rice and *Monascus* pigment) was studied. A total of 18 samples were analyzed by HPLC for citrinin. The results revealed that 11 samples were negative for containing citrinin and 7 samples were positive for containing citrinin with the levels between 0.10 and 594 μg/kg (Table 5).

**Table 5.** Detection results of citrinin in 18 samples

| No. | Sample | Lot No. | Content of Citrinin, μg/kg |
|---|---|---|---|
| 1 | Xuezhikang | 20100811 | undetected |
| 2 | Xuezhikang | 20100901 | undetected |
| 3 | Xuezhikang | 20101108 | undetected |
| 4 | Xuezhikang | 20101211 | undetected |
| 5 | Xuezhikang | 20110103 | undetected |
| 6 | Xuezhikang | 20110111 | undetected |
| 7 | Xuezhikang | 20110201 | undetected |
| 8 | Red yeast rice 1 | 20100820 | 0.97 |
| 9 | Red yeast rice 2 | 20100612 | 1.32 |
| 10 | *Monascus* pigment powder 1 | 20100817 | 594 |
| 11 | *Monascus* pigment powder 2 | 20100718 | 122 |
| 12 | Functional red yeast rice powder 1 | 20090627 | 0.10 |
| 13 | Functional red yeast rice powder 2 | 20090916 | 4.04 |
| 14 | Functional red yeast rice powder 3 | 20090810 | 5.41 |
| 15 | Red yeast rice health food 1 | 20100320 | undetected |
| 16 | Red yeast rice health food 2 | 20100412 | undetected |
| 17 | Red yeast rice health food 3 | 20100501 | undetected |
| 18 | Red yeast rice health food 4 | 20100530 | undetected |

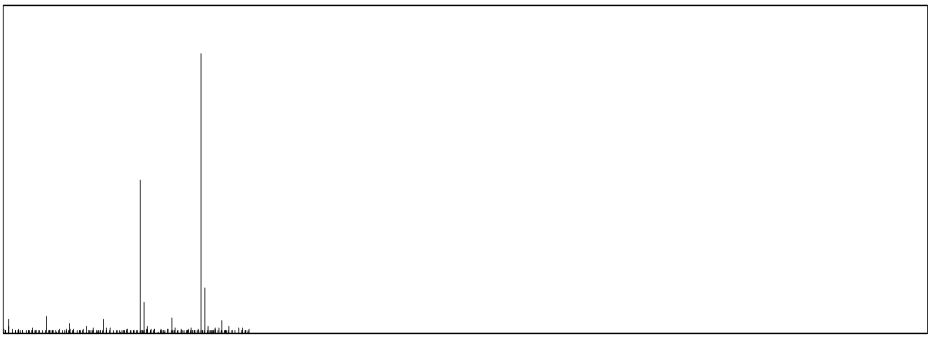
*LC-MS/MS analysis*

Chromatographic separation was modified based on the HPLC condition. The same column was used and the mobile phase A was acetonitrile and mobile phase B was 0.4% formic acid. The gradient eluting system is shown in Table 6. The injection volume was 20 μL. The flow rate was 1.0 mL/min, with the flow rate split down to 0.25 mL/min into the MS source.

**Table 6**. Gradient elution system in LC-MS/MS detection

| Time, min | A, % | B, % |
|---|---|---|
| 0 | 45 | 55 |
| 16 | 45 | 55 |
| 17 | 100 | 0 |
| 25 | 100 | 0 |
| 26 | 45 | 55 |
| 35 | 45 | 55 |

MS analysis was performed by employing the ESI positive ion mode in mass range of 50-2000. High purity nitrogen was used as nebulizer and auxiliary gas. The mass parameters were optimized to the following values: hexapole $R_f$ (200.0 VPP); collision $R_f$ (100.0 VPP); pre pulse storage (5.0 μs); collision energry (7.0 eV); quadrupole ion energy (5.0 eV); nebulizer (0.8 bar); dry gas (5.0 L/min); dry temperature (180 $^{o}$C). The LC-MS/MS chromatograms for the citrinin and xuezhikang are displayed in Figure 3 and 4. In the LC-MS/MS analysis of xuezhikang, it is supposed to be no peak detected at the retention time of citrinin.



## Conclusion

The proposed reverse phase HPLC method has been evaluated over the linearity, precision and recovery and proved to be convenient and effective for the detection of citrinin in *Monascus*-fermented products as well as other routine quality control procedure. Under the reported HPLC conditions, a detection limit of citrinin achieved was as low as 0.187 ng/mL. Quantitative analysis of citrinin in xuezhikang using the proposed protocol suggested that there were no detectable citrinin and the result has been double-confirmed by LC-MS/MS. The method can also be applied for routine quality control analysis of citrinin in *Monascus*-fermented products.

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