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HPLC Analysis of Citrinin in Red Yeast Rice

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Abstract A new and sensitive method for determination of citrinin has been developed. The method includes stability analysis of citrinin, extraction and treatment of sample and reversed-phase HPLC analysis. The results suggested that citrinin was an instable compound, which chemical conversion was strongly dependent on the solvent composition and the temperature. Sample treatment for citrinin trace analysis must control low temperature operation strictly, and suitable downstream process could decrease the content of citrinin in red yeast rice.

Keywords: citrinin, HPLC, red yeast rice, stability

Introduction

Citrinin is a toxic secondary metabolite first isolated from *Penicillium citrinum* Thom. It was also produced by other species of *Penicillium* and *Aspergillus* (1). On account of its powerful antibacterial effect, citrinin was investigated as an antibiotic, but toxicity studies showed that this secondary metabolite acted in animals as a nephrotoxin, damaging the proximal tubules of the kidney. Citrinin was found to disrupt renal function in all species in which it was tested (2). Therefore, it was suggested that it could also be implicated, together with other mycotoxins.

Red yeast rice, produced by fermenting *Monascus* species on steamed rice and extensively used in Chinese foods, is one paradigm of traditional foods consumed in Asia. Clinical observations clearly showed that functional red yeast rice had the ability of lowering blood-lipid levels in animal models and in humans (3, 4). However, monascidin A was isolated and identified from various species of *Monascus* (5). Mass spectroscopy analysis indicated that it was identical in structure to citrinin (5, 6). Contamination of citrinin restricted the further development of traditional *Manascus* products and the usage of functional *Monascus* products as cholesterol-lowering drug in the world-wide.

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While data on citrinin were limited, the reason for lack of data might be analytical problem (7), from the chemical-analytical point of view, it was recommended that the chelating ability, the effects of pH and temperature on citrinin properties were considered (8). In addition, citrinin was known to form the citrinin H_1 toxin, built up of two citrinin molecules, at temperatures above 100°C (9). Common methods used to analyze citrinin were thin-layer chromatography (TLC) (10), high-performance liquid chromatography (HPLC) with UV (11, 12) or fluorescence detection (FD) (13, 14) as well as enzyme immunoassays (15). Detection limits for citrinin from the above-cited literature were in the range 0.01-22.5 ng/g for FD and 2-20 ng/g for UV detection.

It was the goal of this work to study the liquid chromatographic properties and stability of citrinin in detail, in order to develop an accurate and sensitive method to determine citrinin in *Monascus* product. In case of decreasing and controlling citrinin content during *Monascus* processing, we conducted a series of experiments including HPLC analyses of commercial *Monascus* extracts.

Materials and Methods

Materials The commercial samples of *Monascus* fermented rice were obtained from various companies. Parts of the samples were cultured in our laboratory, which were produced in the traditional way, by culturing *Monascus ruber* on steamed rice. Commercial pure citrinin was obtained from Sigma (St. Louis, MO, USA). All solvents used in chromatography were of HPLC grade; solvents used for spectroscopy were of spectroscopic grade, all other reagents used were of analytical grade. Trifluoroacetic (TFA) was purchased from Sigma (St. Louis, MO, USA). Water was of Milli-Q quality.

Preparation of standard and samples Stock solution of citrinin was prepared by dissolving 1.0 mg citrinin in 5 mL acetonitrile aqueous solution. It was stored at −20°C in the dark. Stock solution of citrinin standard was gradient diluted as below: 200 μg/mL, 20 μg/mL, 10 μg/mL, 2 μg/mL, 500 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL.

Ten microliter citrinin stock solution (20 μg/mL) was pipetted into 1.5 mL eppendorf tube, was diluted to hundred

microliter with water, acetonitrile, methanol, ethanol and acetone, respectively, and then vaporized in oven at 60°C for 6 hr. Every sample was recovered to original concentration with original solvent.

In addition, hundred microliter citrinin acetonitrile aqueous solution of 2 μg/mL was pipetted into 1.5 mL eppendorf tube, and then was heated in oven at 60°C for 6 hr under sealed and unsealed condition, respectively. Hundred microliter citrinin acetonitrile solution of 2 μg/mL was volatilized to dryness at room temperature. Every sample was recovered to original concentration with acetonitrile aqueous solution.

Red yeast rice powder samples were accurately weighted 200 mg±0.2 mg, and put into 1.5 mL eppendorf centrifuge tube, extracted with 1 mL acetonitrile / water (60:40 v:v) by ultrasonic power at 20°C for 20 min, the extraction were centrifuged at 12,000× g for 5 min at 4°C. The clear supernatants were stored at −20°C for HPLC analysis use. Twenty microliter supernatant were directly injected into the HPLC system. Every above-mentioned treatment was repeated up to 3 times.

Microorganism and growth conditions The strain used was one of *Monascus ruber* obtained from China, producing lovastatin. The stock culture was maintained on difco potato dextrose agar (PDA). Spores were prepared by growth on PDA slants for 8 days at 30°C, and were harvested and washed with sterile water. A suspension of 10^8 spores was used to inoculate a 250 mL baffled Erlenmeyer flask containing 100 mL medium. This inoculum was cultured at 30°C for 2 days and then transferred to 250 mL baffled Erlenmeyer flask containing 100 mL synthetic medium. The cultures were incubated in shaking incubator at 250 rpm at 30°C for 10 days. The solid culture on steamed rice was carried out in plastic mushroom bottles at 32°C for 15 days, solid medium was consisted of 100 g rice, 14 g soybean powder, 2 g sucrose, 1 g yeast extract.

Instruments and Chromatographic conditions The HPLC system was System Gold® (Beckman, Fullerton, CA, USA) equipped with 128 solvent module and 168 Detector and a power supplier, a 7725 auto sampler and an on-line degassing instrument. ODS column (250×4.6 mm, 5 μm) from Sphenomenex was used. The 168 detector was equipped with a photo-diode array detector. Gold™ Nouveau Chromatography Station was used for system control, data collection and analysis.

Both gradient and isocratic elution were used, according to the experimental requirements. General conditions were listed below and apply unless otherwise noted. For isocratic elution, the mobile phase consisted of acetonitrile/water containing 0.05% TFA (62:38, v/v) with a flow-rate of 0.5 mL/min. Citrinin stability trials were carried out in the manner of isocratic elution. Stepped gradient elution program was achieved as follows: 0.5 mL/min for 10 min; 0.2 mL/min for 10 min; 0.5 mL/min for 10 min, the samples were carried out in the manner of stepped gradient elution.

UV detection was performed with UV detector (Beckman, Fullerton, CA, USA) set at 334 nm, citrinin exhibit UV absorption (Citrinin: λ_{Max}^{CH3CN} (nm) = 334 [this work]). The UV diode array detection range was set from 200 nm to 600 nm.

Precision was evaluated by injecting twenty microliter of citrinin standard solution (2000 ng/mL) containing 40 ng citrinin in a isocratic run. All precision and reproducibility analysis were run on Sphenomenex column.

Results and Discussion

Chromatographic evaluation of stability analysis for citrinin Reproducibility of retention time and peak area over a three-week period resulted in relative standard deviations (RSD) for citrinin of 1.1% (n=5) and 2.3% (n=7), respectively, nine consecutive injections revealed no variation in retention time and an RSD of 1.1% for the peak area of citrinin, which indicated that this HPLC system had a very high accuracy and precision.

Its linear range was from 200 ng/mL to 20 μg/mL and the linear regression equation was: Y=7.15957e-006x Area+0. The correction coefficient was 0.999 with a signalto-noise ratio of 2. The minimum detectability of citrinin in this HPLC system was 200 ng/mL containing 4 ng/20 μL of injection as absolute amount. Its chromatogram of gradient concentration citrinin and its minimum detectability were shown in Fig. 1.

Chromatographic evaluation of sample analysis Typical chromatogram of *Monascus* product as Fig. 2, the peak

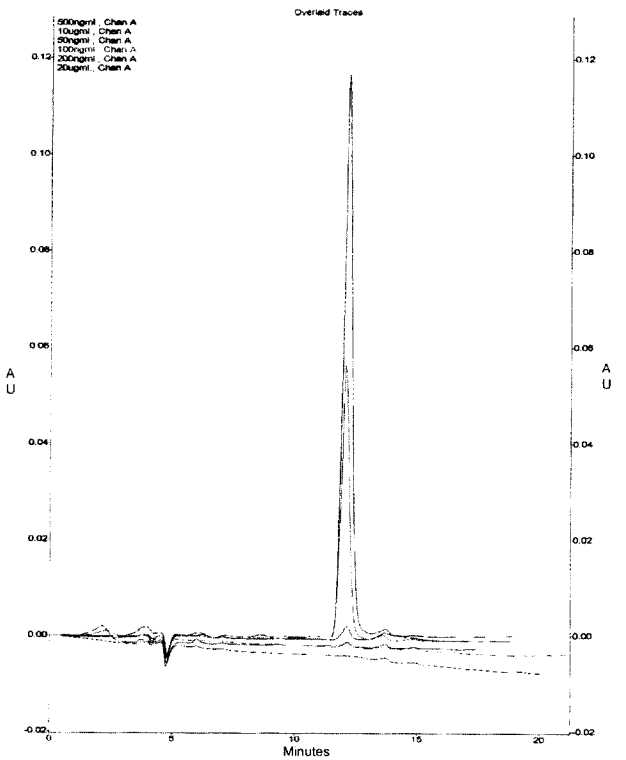

Fig. 1. Chromatogram of gradient concentration citrinin.

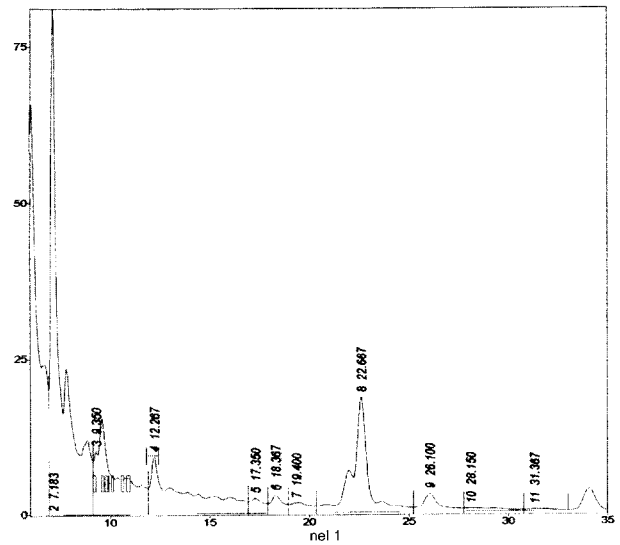

Fig. 2. Typical chromatogram of *Monascus* products contaminated with citrinin.

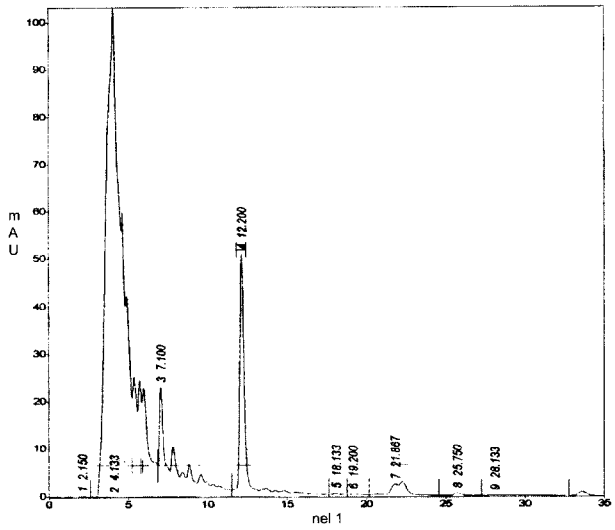

Fig. 3. Chromatogram of sample plus standard.

of retention time 12.26 min was that of citrinin.

Recovery rate trails were carried out on sample lovastatin1, which contaminated with citrinin. 2 μg, 4 μg and 6 μg citrinin was added into hundred microliter sample solution which known citrinin concentation, respectively. The results indicated that recovery rate was 98%, 105%, 93%, respectively. Its average recovery rate was 96.75%. HPLC chromatogram of sample plus standard was shown in Fig. 3. The peak of retention time 12.2 min was overlap peak of standard citrinin and sample citrinin.

Effect of temperature on stability of citrinin Lately study was carried out by Hirota *et al.* (16), they examined and isolated the decomposition products after heating, and a major product was elucidated as citrinin H_2. HPLC analysis of citrinin after heating under various conditions indicated that citrinin H_2 was mainly yielded from citrinin. Our research (Table 1) suggested that temperature was an important sensitive factor for stability of citrinin. Under room temperature and various heating conditions, citrinin could be converted into other compounds; under low temperature (less than 4°C), its stability could be preserved. Thus, HPLC determination of citrinin must control low temperature operation strictly, decrease the time of sample existing under room temperature as less as possible, and avoid thermal operation procedure in case of gaining negative or wrong results.

Effect of solvents on stability of citrinin Completely different behaviors were observed for citrinin in different

Table 1. Effect of temperature on stability of citrinin

Sample treatments (in acetonitrile solution)	Original concentration (μg/mL)	Final concentration (μg/mL)	Transformation ratio (%)
1[a)]	2.000	0.458±0.002	77.100%
2[b)]	2.000	0.958±0.006	52.100%
3[c)]	2.000	0.933±0.005	53.360%
4[d)]	2.000	2.000±0.080	0.000 %
5[e)]	2.000	2.000±0.040	0.000 %

[a)] Heated without sealing in oven at 60°C.
[b)] Heated with sealing in oven at 60°C.
[c)] Volatilized to dry at room temperature.
[d)] Preserved at 4°C in refrigerator.
[e)] Preserved at −20°C in refrigerator.

Table 2. Effect of solvents on stability of citrinin

Sample treatments	Original concentration (μg/mL)	Final concentration (μg/mL)	Transformation ratio (%)
H_2O	2.000	0.816±0.001	59.200%
Ethanol	2.000	0.663±0.003	66.850%
Acetonitrile	2.000	0.458±0.009	77.100%
Methanol	2.000	0.3765±0.008	81.400%
Acetone	2.000	0.3225±0.004	83.870%

solvents. Citrinin was converted easily in solvent solution, and the conversion was strongly dependent on solvent composition and temperature. These data (Table 2) in the manner of HPLC quantitative analysis supported the research results of Poupko *et al.* (17).

Sample treatment For development of this method, we carried out various pre-cleaning steps by TLC and C_{18} sep-pak cartridges. However, when TLC was used according to the method (10), the sensitivity of the method decreased. And when C_{18} sep-pak cartridges was used, the results suggested that citrinin was distributed into water elution and organic solvent elution, quantitative analysis could not be carried out accurately. These results were omitted in this paper. Complex and long time pre-treatment caused the loss of citrinin content, meanwhile, existed several effect factors for stability of citrinin. Thus, HPLC quantitative and qualitative analysis of citrinin should be simple and rapid. So the extracts in our research were directly subjected to HPLC system.

Analysis for samples Citrinin could be detected in some commercial *Monascus* products obtained from different sources in variable concentrations ranging from 0.00 to 1.97 μg/g. The amounts of citrinin detected in the different samples were summarized in Table 3.

Downstream process of red yeast rice contaminated with citrinin Since citrinin could be detected in some commercial *Monascus* preparations as well as our cultured samples, in addition, citrinin owns thermal instability, downstream process experiments (Table 4) of *Monascus* solid state cultures were carried out .

The presented studies provide further evidence that thermal treatment could decrease the content of citrinin in red yeast rice. These data supported the research results by Chinese researchers (18). Contamination of *Monascus* product with citrinin should be avoided by traditional fermentation technology firstly, once *Monascus* product was contaminated with citrinin, thermal treatment should be done to decompose citrinin. However, toxicity of derivate compound and adductive compound of citrinin need be further studied. Meanwhile, standardized manufacturing practices should be established for red yeast rice sold as a dietary supplement in order to ensure equivalence of content of active ingredients in preparations for selling to the public and to limit the production of unwanted byproducts of fermentation such as citrinin.

Table 3. Amount (μg/g) of citrinin detected in different samples

Samples No.	Citrinin detected (μg/g)	Samples origin
1	0.000±0.000	3.4633 (1)*
2	0.000±0.000	3.4633(5) *
3	11.974±0.070	Cholestin (USA.)
4	0.000±0.000	JSM (Korea)
5	4.679±0.035	Lovastatin 1 *
6	5.384±0.006	Lovastatin 2*
7	0.000±0.000	Lovastatin 3*
8	7.600±0.040	FM011*
9	0.000±0.000	1.4% taiwan (Taiwan, China)
10	2.127±0.003	Pigment rice (Fujian, China)

*Samples were cultured in our lab.

Table 4. Effect of thermal process on *Monascus* products contaminated with citrinin

Treatments	Original concentration (μg/g)	After process concentration (μg/g)	Transformation ratio (%)
1 [a]	5.384	5.369±0.040	0.000%
2 [b]	5.384	0.000±0.000	100.00%
3 [c]	5.384	2.546±0.013	52.740%
4 [d]	5.384	5.027±0.032	6.630%

[a] No process.
[b] Sterilized in autoclave at 121°C for 20min.
[c] Dry in oven at 60°C.
[d] Dry under sun light.

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