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Analytical Methods

Simultaneous determination of two *Monascus* metabolites in red yeast rice by HPLC using fluorescence detection

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ABSTRACT

A reversed-phase HPLC method with fluorescence detection for the determination of two *Monascus* metabolites, monasfluore A (MFA) and monasfluore B (MFB), in red yeast rice was carried out. Optimum conditions for the extraction and chromatographic separation were investigated. The method was validated through the following performance criteria: linearity, stability, limit of detection (LOD), quantification (LOQ), etc. This assay was successfully used for determination of the MFA and MFB in 20 samples inoculated from different *Monascus* sp. The results revealed that significant variations were demonstrated in the contents of the MFA and MFB in these samples. The high contents of both MFA and MFB in sample 13 were found to be 81.400 and 26.300 mg g^{-1}, respectively. The low contents of both MFA and MFB in sample 14 were also found to be 0.010 and 0.003 mg g^{-1}, respectively.

1. Introduction

The filamentous fungi of the genera *Monascus*, which is a food fungus, has been widely applied in Asia for centuries as a source of pigments for the colouring of traditional foods. Many metabolic derivatives, such as the pigments, monacolins, γ-aminobutyric acid (GABA), dimerumic acid and ethanol can be produced by *Monascus* sp. (Juslová, Martínková, & Kren, 1996; Ma et al., 2000).

Red yeast rice, also known as *Monascus* fermented rice, red koji, anka rice, angkak and ben-koji, is produced by growing *Monascus* sp. on cooked rice to produce a red-coloured product (Liu, Wu, & Tan, 2010; Tseng, Chen, & Lin, 2000).

Since several centuries, red yeast rice is being used for colouring, flavouring and preservations of foods in East Asia (Mohan Kumari, Akhilender Naidu, Vishwanatha, Narasimhamurthy, & Vijayalakshmi, 2009). In China, red yeast rice was also used as a Chinese medicine to strengthen the spleen, promote digestion, eliminate dampness and phlegm, promote blood circulation and remove blood stasis. Moreover, in recent studies, it has been confirmed that red yeast rice has some medicinal properties, such as being an antihypertensive, hypocholesterolaemic, antimicrobial, antioxidative and anticarcinogenetic agent (Wang & Lin, 2007). However, the pattern of natural products in red yeast rice is extremely complex, and it consists of a wide range of compounds of varying polarity. In the past decade, some new metabolites such as Monascodilone (Wild, Toth, & Humpf, 2002), Monascopyridines A and B (Wild, Toth, & Humpf, 2003), Monascopyridines C and D (Knecht, Cramer, & Humpf, 2006), Xanthomonascins A and B (Akihisa et al., 2005), Monankarins A–F (Hossain, Okuyama, & Yamazaki, 1996), monaspilosin, monaspiloindole and monaspyranoindole (Cheng, Wu, Chen, & Yuan, 2008) from red yeast rice have been isolated and their chemical structures have been characterised. The pharmacological and toxicological effects of most secondary metabolites are still unknown or unclear (Hossain et al., 1996; Knecht et al., 2006), and there are even more unidentified metabolites in the red yeast rice. Recently, our group reported the extraction, purification and characterisation of the structures of molecules involved in two new fluorescent compounds in red yeast rice (Huang, Xu, Li, & Li, 2008). The two Monascus metabolites are named as monasfluore A (MFA) and monasfluore B (MFB), respectively, and their chemical structures are shown in Fig. 1a. The two *Monascus* metabolites were also reported in a literature (Zheng, Xin, & Guo, 2009). However, there are no detailed studies available concerning the distribution of MFA and MFB in red yeast rice or the analytical methods used for their determination in red yeast rice samples. Development of a simple and rapid HPLC method for the determination of MFA and MFB in red yeast rice is therefore necessary and would be valuable for further study.

The aim of this study was to establish a reliable HPLC method for simultaneous determination of the two *Monascus* metabolites in red yeast rice. Optimisation of the extraction solvent and HPLC method were followed by a comprehensive validation study, which covered linearity, repeatability, stability, accuracy, limits of detection and quantification.

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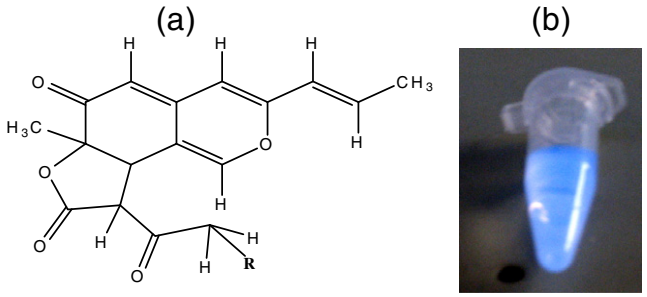

Fig. 1. Chemical structure of MFA and MFB (a) and strong blue fluorescence under UV 365 nm lamp (b).

2. Materials and methods

2.1. Standards and reagents

HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionised water was obtained with an in-house Milli-Q Plus System (Millipore, Inc., Billerica, MA, USA) at 18.20 MΩ cm; this deionised water is referred to as "water" hereafter. Before analysis, the mobile phase was filtered through a 0.45 μm nylon filter from Millipore (Millipore, Inc., Billerica, MA, USA) under vacuum. Chemical standards monasfluore A (MFA) and monasfluore B (MFB) were isolated from red yeast rice in our laboratory (Huang et al., 2008), and their purities (>98.5%) and structures determined by HPLC, MS, and NMR. According to the data their structures were presented in Fig. 1a. All other chemicals were of analytical grade from ChromTech (Shanghai, China) and were not further purified.

2.2. Standard solutions

MFA and MFB standard (10.00 mg) were weighted, respectively, with a Sartorius electronic balance (Göttingen, Germany) (sensitivity = 0.01 mg). Stock solutions (1.00 mg mL^{-1}) of MFA and MFB were prepared by dissolving 10.00 mg in 10 mL of methanol, respectively. They were kept in brown glass vials in the freezer (−18 °C) and were stable at least 3 months. The stock solutions were serially diluted, mixed and used for preparation of working standard solutions.

2.3. Sample preparation

The red yeast rice samples were prepared according to our previous work (Huang et al., 2008). The strains *Monascus*, such as AS3.976, AS3.978, AS3.444, AS3.4451, AS3.4452, AS3.4453, AS3.2636, etc., were obtained from the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). After 5.0 mL of distiled water was put into a strain slant and vortexed, the spore solutions were collected. Seed cultures were grown in 500.0 mL baffled flasks containing 75.0 mL of malt extract agar medium that had been inoculated with 5.0 mL of the spore solutions. Flasks were then cultivated for 36 h at 30 °C and 200 rpm on a rotary shaker.

To obtain 20 of the red yeast rice samples, 500.0 mL baffled flasks each containing 100.00 g steamed rice was inoculated with 2.0 mL of the seed cultures different *Monascus* sp., respectively, and then cultivated for 2 weeks at 30 °C (humidity 50–60%). These red yeast rice products were dried at 40 °C and milled to homogeneous powders, and then sieved through a No. 60 mesh after solid state fermentation. From above the process, 20 of red yeast rice samples were obtained, and three replicates were done for each the red yeast rice product.

2.3.1. Reference standard preparation

Reference standard of MFA and MFB were prepared according to the method described our previous work (Huang et al., 2008). MFA and MFB were further purified by semipreparative HPLC on a 150 × 9.4 mm i.d., 5 μm Zorbax SB-C_{18} column (Agilent, Santa Clara, CA, USA) using an isocratic elution using water/methanol (30:70, v/v) ,and a flow of 2.50 mL/min.

2.3.2. Samples extraction

A 1.00 g aliquot of the red yeast rice powder was weighed into an extraction/centrifugation tube and suspended in 10.0 mL of methanol. The tubes were sonicated for 20 min at room temperature and centrifuged for 20 min at 10,000 rpm. The supernatant was decanted into a clean dry vial and the residue re-extracted three more times with methanol (5.0 mL). The extracts were combined and diluted appropriately with methanol, and then the extract was filtered through a 0.45 μm syringe filter for the HPLC analysis. Three replicate extractions for each red yeast rice sample were carried out on at least three different days.

2.4. Optimisation

2.4.1. Chromatographic separation

The parameters varied for optimisation of the chromatographic conditions were: (a) compositions of mobile phase (such as methanol–H_2O and acetonitrile–H_2O), (b) detection wavelength (such as UV/vis (λ_{max} = 370 nm), and fluorescence (λ_{ex} = 396 nm and λ_{em} = 460 nm)).

2.4.2. Extraction conditions

The optimisation of sample extraction included varying (a) compositions of extraction solvents (different proportions of methanol–H_2O), (b) sonication time Different extraction mixtures and sonication time were examined for the extraction efficiency of red yeast rice (1.00 g) that had been spiked with MFA and MFB standards (0.60 mg), respectively. Extraction efficiency was calculated as: Extraction efficiency (%) = 100 × ([red yeast rice + spike] − [red yeast rice + blank]) × ([spike])$^{-1}$.

2.5. HPLC analysis conditions

The HPLC system consisted of a Waters 510 solvent delivery pump (Waters, Milford, MA, USA), a 7725 manual injector system equipped with a 20 μL loop, a model 2475 multi-wavelength fluorescence detector (Waters, Milford, MA, USA), and equipped with Echrom98 chromatography manager for integration (Dalian Elite Analytical Instruments Co. Ltd., Dalian, China). Fluorescence detection was performed with a 2475 multi-wavelength fluorescence detector with λ_{ex} = 396 nm and λ_{em} = 460 nm. Chromatographic separation was achieved at room temperature using a 250 × 4.6 mm i.d., 5 μm Symmetry C_{18} column (Waters, Milford, MA, USA), with isocratic elution of acetonitrile–H_2O (80:20, v/v) at a flow rate of 0.80 mL min^{-1}. Aliquots of 20 μL standard or samples solutions were injected into the HPLC for the determination. All injections were repeated at least three times.

2.6. Method validation

The quantitation was accomplished through a calibration curves with six concentration levels in the range of 0.50–10.00 ng mL^{-1} for MFA and 0.25–5.00 ng mL^{-1} for MFB, and for each level of concentration three replications were performed. The linearity of the method was investigated by calculation of

the regression line by the method of least squares and expressed by the correlation coefficient (r). The sensitivity of the method was evaluated by estimating the limit of detection (LOD) and the limit of quantitation (LOQ) at a signal to noise ratio (S/N) of 3 and 10, respectively, for the standard solutions. Recoveries were determined a red yeast rice sample (1.00 g) spiked at two different levels (0.40 and 1.00 mg) of both MFA and MFB, and subjected to extraction as described above. The relative standard deviation (RSD) at two concentration levels were determined ($n = 3$ replicates per concentration level and analysed in three independent analytical runs).

3. Results and discussion

3.1. Optimisation of chromatographic conditions

In order to obtain a good separation and resolution of MFA and MFB in the chromatograms, various compositions of mobile phase (such as methanol–H_2O (v/v): 60:40, 70:30, 80:20, 90:10, and acetonitrile–H_2O (v/v): 60:40, 70:30, 80:20, 90:10) were tried to obtain chromatograms with good resolution of adjacent peaks. The results indicated that the analysis time was prolonged with the decrease of methanol or acetonitrile concentration. Finally, an

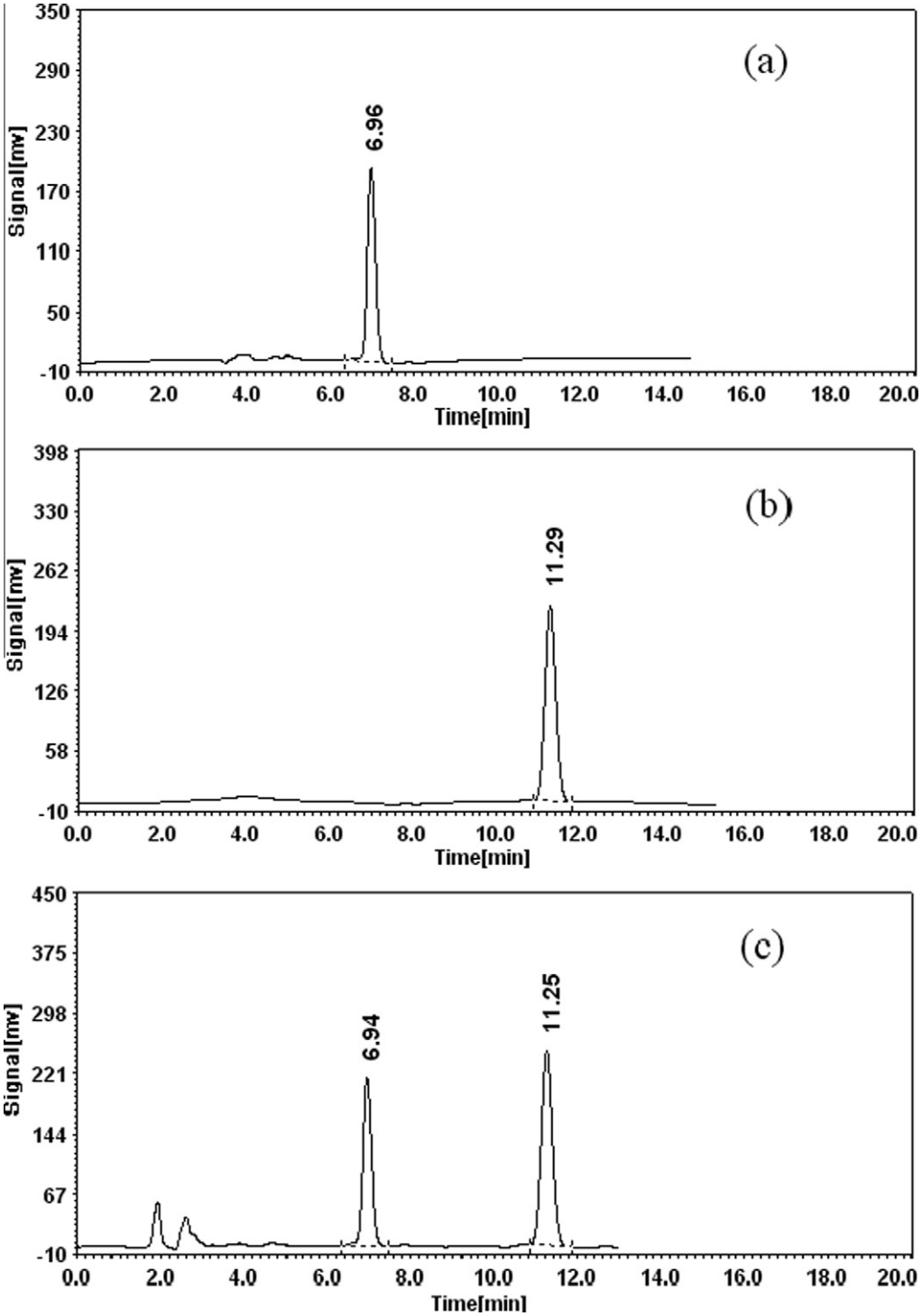

Fig. 2. Chromatograms of MFA (a) and MFB (b) standard, and MFA and MFB in red yeast rice sample (c) by HPLC.

isocratic solvent system composed of acetonitrile–H_2O (80:20, v/v) was selected as a mobile phase, since it afforded a good separation and resolution of target peaks. The MFA and MFB had strong blue fluorescence under UV 365 nm lamp (Fig. 1b), and they had similar fluorescence spectra and ultraviolet/visible spectra (maxium UV/vis wavelength at 370 nm) , with maximum excitation and emission at 396 nm and 460 nm, respectively (Huang et al., 2008). When response values of MFA and MFB were compared by ultraviolet and fluorescence detectors connected in series, the response values of the latter were more than 100-fold higher than that of the former (Huang et al., 2008). In this study, the excitation wavelength of 396 nm and emission wavelength 460 nm were selected for detection of MFA and MFB, since they showed good sensitivity of the target peaks. The typical chromatographic profiles of MFA and MFB standard and actual sample are shown in Fig. 2. No interference was observed for target compounds in the chromatograms of the samples.

3.2. Optimisation of sample extraction

In order to achieve the optimal extraction conditions, variables involved in the extraction procedure such as extraction solvents, extraction time were investigated.

In this study, different proportions (50%, 60%, 70%, 80% and 100%) of methanol were extracted as described above to assess their extraction efficiency. Of the combinations investigated, using pure methanol was more suitable than the others. Using pure methanol gave more satisfactory extraction efficiency, the MFA and MFB were at least 85% extracted. Moreover, extraction with an ultrasonic processor was common practice for the exhaustive extraction of *Monascus* metabolites from red yeast rice samples for quantitative analysis. This extraction method was therefore used in this work. The effect of time on the extraction efficiency was evaluated by using pure methanol for 5, 10, 20, 30 and 60 min, respectively. The results showed that using 20 min of sonication was more satisfactory extraction efficiency than the others, and the average extraction efficiency of the MFA and MFB was about 84.80% and 83.60%, respectively.

3.3. Method validation

3.3.1. Calibration curves, limits of detection and quantification

The calibration curve of the individual standards was constructed using six concentrations ($n = 3$) by plotting peak areas against the concentration of analytes. Good linearity was observed in calibration curves over the concentration ranges investigated. LOD of MFA and MFB were 0.28 and 0.12 ng mL^{-1}, respectively, and LOQ were 0.93 and 0.41 ng mL^{-1}, respectively. The results are summarised in Table 1.

3.3.2. Repeatability and precision

The repeatability of the peak areas and retention times for MFA and MFB were studied by repeatedly injecting mixtures containing 2.00 ng mL^{-1} MFA and MFB, respectively. It was observed that both retention times and peak areas were reproducible and all the relative standard deviation (RSD) values obtained were satisfactory (<3.0%). The reproducibility over different days was checked by injecting the different standard solution 1.25, 2.50 and 5.00 ng mL^{-1} for MFA ($n = 3$), and 0.50, 1.00 and 2.00 ng mL^{-1} for MFB ($n = 3$), respectively, over three days. The RSD for peak area ranged from 2.13% to 2.59% for MFA, and from 2.10% to 2.92% for MFB.

3.3.3. Stability

For stability test, the same sample solution was analysed every 2 h over 10 h at the room temperature. The RSD of contents of MFA and MFB in the same sample was 2.27% and 2.58%, respectively, which indicated that the sample was stable over 10 h under the experimental conditions.

3.3.4. Recovery

To determine the recoveries, a red yeast rice sample spiked at 0.40 and 1.00 mg g^{-1} of MFA and MFB were extracted and analysed in triplicate over 3 consecutive days. As shown in Table 2, the resultant RSD for these studies were found to be 3.60–4.50% for MFA and 4.70–5.20% for MFB with a corresponding percentage recovery value of 85.30–87.60% and 82.30–85.80%, respectively. It was indicated that the extraction method was efficient for determination of the MFA and MFB in red yeast rice.

Table 2
Recovery data for MFA and MFB in red yeast rice.

Compound	Original (mg)	Spike (mg)	Found (mg)	Mean recovery (%)	RSD (%) ($n = 3$)
MFA	0.50	0.40	0.763	85.3	4.5
		1.00	1.305	87.6	3.6
MFB	0.20	0.40	0.486	82.3	5.2
		1.00	1.008	85.8	4.7

Table 3
Content (mg g^{-1}) of MFA and MFB in different red yeast rice samples ($n = 3$).

Samples	Content of MFA, mg g^{-1}, (mean ± SD)	Content of MFB, mg g^{-1}, (mean ± SD)
1	1.290 ± 0.030	0.170 ± 0.040
2	1.530 ± 0.100	0.240 ± 0.090
3	9.700 ± 0.360	2.700 ± 0.430
4	3.050 ± 0.170	0.840 ± 0.080
5	4.220 ± 0.180	1.540 ± 0.090
6	1.410 ± 0.050	0.260 ± 0.060
7	0.110 ± 0.040	0.030 ± 0.010
8	0.390 ± 0.060	0.170 ± 0.060
9	0.500 ± 0.080	0.200 ± 0.030
10	34.600 ± 2.070	11.400 ± 1.680
11	0.040 ± 0.010	0.020 ± 0.010
12	0.020 ± 0.010	0.010 ± 0.003
13	81.400 ± 3.920	26.300 ± 2.430
14	0.010 ± 0.003	0.003 ± 0.001
15	1.290 ± 0.130	0.210 ± 0.030
16	0.970 ± 0.110	0.360 ± 0.110
17	0.260 ± 0.060	0.040 ± 0.010
18	0.980 ± 0.150	0.160 ± 0.050
19	11.100 ± 0.950	4.240 ± 0.600
20	0.650 ± 0.170	0.100 ± 0.030

Table 1
Regression equations, LODs and LOQs for MFA and MFB.

Compound	Linear regression	Linear range (ng mL^{-1})	r	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
MFA	$Y = 772.5X - 289.2$	0.50–10.00	0.9994	0.28	0.93
MFB	$Y = 2376.6X + 1346.4$	0.25–5.00	0.9972	0.12	0.41

Where, X is the concentration of analytes and Y is the peak area.

3.4. Sample analysis

The method was applied to red yeast rice obtained with different *Monascus* by solid state fermentation. In total, 20 samples were analysed, and the content of MFA and MFB ranged from 0.010 to 81.400 mg g^{-1}, from 0.003 to 26.300 mg g^{-1} respectively. The details were summarised in Table 3. According to the results, some red yeast rice samples were rich in MFA and MFB, but there was a large variation in MFA and MFB content amongst the samples.

4. Conclusions

In this study, a HPLC method has been established for the quantitative analyses of MFA and MFB in red yeast rice. The method is simple, sensitive and reliable. Comparing the results of the analyses, we found a substantial variation in the MFA and MFB content of the red yeast rice samples tested. The high contents of both MFA and MFB in red yeast rice (sample 13) were found to be 81.400 and 26.300 mg g^{-1}, respectively. The low contents of both MFA and MFB in red yeast rice (sample 14) were also found to be 0.010 and 0.003 mg g^{-1} respectively. It will be interesting in future research to investigate the factors led to the variation in the MFA and MFB content of different the red yeast rice samples.

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