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Identification and chemical profiling of monacolins in red yeast rice using high-performance liquid chromatography with photodiode array detector and mass spectrometry

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Abstract

Monascus purpureus-fermented rice (red yeast rice) was one of the food supplements that had the ability of lowering the blood-lipid levels, and monacolins have been proved to be main active constituents. In total 14 monacolin compounds such as monacolin K (mevinolin), J, L, M, X, and their hydroxy acid form, as well as dehydromonacolin K, dihydromonacolin L, compactin, 3α-hydroxy-3,5-dihydromonacolin L, etc. were identified in red yeast rice, using high-performance liquid chromatography with photodiode array detector and tandem mass spectrometry. A chemical fingerprint profiling method to display bioactive monacolins in red yeast rice was established and could be used for the quality control of the target material and its related products. Ten finish products labeled as red yeast rice from different manufacturers in marketing were traced using the chromatographic chemical profiling method, and the results show that only two of them were similar while the other eight were significantly different from the reference red yeast rice. All of these materials including raw material powder and finished products available were quantified and the contents of monacolins were calculated with reference of monacolin K (mevinolin) as the standard.

Keywords: Monacolin; Red yeast rice; Mevinolin; Identification; Chemical profiling; HPLC; LC-MS

1. Introduction

Increased levels of cholesterol and triglycerides are known to be risk factors for developing coronary artery diseases. Lipid-lowering agents that inhibit HMG coenzyme A reductase are now prominent among the drugs of choice for treating hypercholesterolemia. It is another effective way to control cholesterol level

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with diet and food supplements [1]. Red yeast rice, (rice fermented with red yeast *Monascus purpureus)*, was found to be a food product that has the ability of reducing blood-lipid levels in both animal models and in humans [2–5]. Red yeast rice, or Hongqu in Chinese, has been used in China as food or medicine for improving digestion and blood circulation for thousands of years. The modern research has revealed that the main components of *M. purpureus*-fermented red rice contributing to the pharmacology effects involve monacolin-type compounds [6,7]. Since 6-demethylmevinolin (also referred to as compactin, Mevastatin, ML 236B, CS500) was isolated by Endo [8] and by Brown and Smale [9] from *Penicillium citrinum* and *P. brevicompactum*, respectively, a series of monacolin compounds have been found. In particular, monacolin K, also called mevinolin or lovastatin, was first reported from *Monascuc ruber* [10] and, independently, by Alberts et al from *Aspergillus terreus* [11]. Furthermore, monacolin J and monacolin L were isolated and reported in 1985, and then dihydromonacolin L and monacolin X [14] were found and monacolin M [15] was disclosed from *M. ruber*; and monacolin L was also isolated from *A. Terreus* cultures. All of these monacolins were found to be effective as hypocholesterolemic agent. It was reported that these monaoclins were the secondary metabolites of biosynthesis during the fermentation and they were biogenetically related to each other [16–18]. Monacolins were a group of compounds existing in both lactone forms and hydroxy acid forms [19,26]. Seven monacolins were isolated from *M. purpureus*-fermented rice [6]. The monacolins in lacton forms were easily purified and crystallized, so their structures were well elucidated. In contrast, the monacolins in hydroxy acid forms were seldom provided with the detailed structural information, as the isolation and purification of free monacolin hydroxy acid forms were seriously hampered by the fact that they were structurally unstable. To identify the existence of monacolin hydroxy acids, the formation of their ester derivations has been reported [13]. One study [6] mentioned the HPLC analysis of monacolin hydroxy acid, but with no detailed illustration on the data obtained. The liquid chromatography with tandem mass spectrometry has become an indispensable tool to elucidate the structure information without isolation and purification. The objective of this paper was to identify the monacolins in *M. purpureus*-fermented rice existing in both lacton form and hydroxy acids form using technology of hyphenated liquid chromatography and mass spectrometry.

Due to the co-existence of multi-components, quality control and standardization of complex herbal or natural products was a challenging task. An effective way in the research area of herbal or botanical products was to construct the chromatographic chemical profiling also called chromatographic fingerprint [21,22]. Chromatographic profiling could provide the images of chemical components of not only the marker compounds but also other active or potential bioactive, even toxic, constituents. So, in this report, the chemical fingerprint profiling of monacolin compounds in red yeast rice was also addressed by liquid chromatography. The content of monacolins in red yeast rice was estimated as well, referenced to monacolin *K* (mevinolin), one of main monacolins and standard commercially available (Fig. 1).

2. Experimental

2.1. Chemicals, reagents and materials

Methanol, acetonitrile(ACN) and trifluoroacetic acid (TFA), HPLC grade; 95% ethanol and sodium hydroxide, AR grade; de-ionized water was obtained with an in-house Milli-Q water system (Millipore Inc., USA). Monacolin K (also called mevinolin) standard was ordered from Sigma (MO, USA). Five batches of red yeast rice powder were kindly provided by a healthcare company in Shanghai, China. Commercial products labeled as red yeast rice, sample 1 (CP1) capsule, 2 (CP2) capsule, 3 (CP3) capsule, 4 (CP4) capsule, 5 (CP5) tablets, and 6 (CP6) tablets were purchased from US store marketing, products 7 (CP7) tablets and 8 (CP8) capsule from Taiwan, and products 9 (CP9) capsule and 10 (CP10) tablets from China marketing.

2.2. Equipment

Liquid chromatography and mass spectrometry (LC/PDA/MS) were carried out using a system equipped with Survyor LC system (Thermo Finnigan,

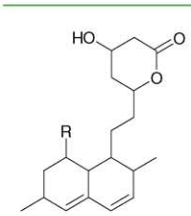
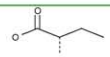
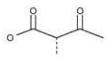
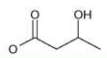
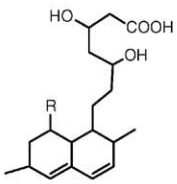
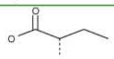
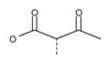
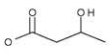
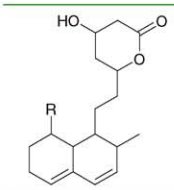
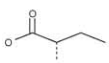
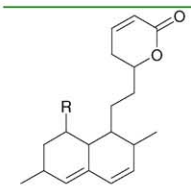
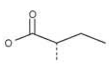
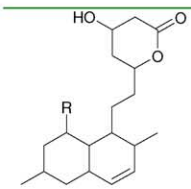
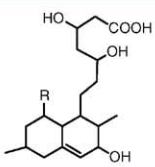
Structure	Name	R	MW	UV (λ_{max})	Ref.
	1. Monacolin K (MK)		404	230, 237, 246	10-12, 20
	2. Monacolin J (MJ)	OH	320	230, 237, 247	13
	3. Monacolin L (ML)	H	304	230, 237, 247	13
	4. Monacolin X (MX)		418	230, 237, 247	14
	5. Monacolin M (MM)		406		15
	1a. MK acid form (MKA)		422		25, 26
	2a. MJ acid form (MJA)	OH	338		
	3a. ML acid form (MLA)	H	322		
	4a. MX acid form (MXA)		436		
	5a. MM acid form (MMA)		424		
	6. Compactin (P1)		390	230, 237, 247	8 ,9
	7. Dehydromonacolin K (DMK)		386		6
	8. Dihydromonacolin L (DML)	H	306		14
	9. 3α-hydroxy-3,5-dihydromonacolin L (HDML)	H	340		23

Fig. 1. Structural data of monacolins in fermented red rice [6,8–15,20,23,25,26].

San Jose, USA) including quaternary pump, on-line degasser, column heater compart, autosampler and photodiode array detector, and a LCQ DECA XP^{plus} mass spectrometer (Thermo Finnigan, San Jose, USA) that consists of an ESI interface and an ion trap mass analyzer. The software for the control of the equipment, and the acquired and treatment of data is Xcalibur1.3 workstation.

The chemical fingerprints were conducted on the HPLC system of Waters 2690 Alliance (Milford, MA, USA), consisting of quaternary pump solvent management system, an on-line degasser and an autosampler. The raw data were detected by 996 photo-diode array detector (PDA), acquired and processed by Waters Millennium32 chromatographic workstation loaded on a Compaq computer. Prior to each run, the HPLC-PDA system was allowed to warm up for nearly 30 min and the baseline was monitored until stable before the samples were injected.

2.3. Sample preparation

About 0.5 g red yeast rice powder, accurately weighed, was transferred to a 20-ml tampon centrifuge tube. The preparations in triplicate were extracted with 10 ml of 75% ethanol for 60 min on an ultrasonic bath and subsequently centrifuged for 10 min at 3000 rpm. This extraction procedure was repeated three times, and the total supernatant was transferred to a 25 ml volumetric flask, adding 75% ethanol to exactly 25.0 ml. The final solution was kept standing for 30 min, and then filtered through a 0.45-μm membrane before injection. For the determination of monacolins in the finished products (tablets or capsules), an amount corresponding to 0.5 g of red rice powder was used for each extraction.

2.4. Preparation of the standard solution

Calibration curves were obtained from purified monacolin K. The purity of the standard was evaluated by HPLC-PDA with detection at 237 nm, by recording the peak purity index via photodiode array detection, and by TLC chromatography. The purity was found to be greater than 99%.

Monacolin K (5 mg, weighed accurately) was dissolved in 75% ethanol in a 25 ml low actinic volumetric flask. This solution was diluted with 70% aqueous ethanol to obtain standard solutions for the calibration curve in a range of 0.2–20 ng on column ($n = 6$), and a 20-μl aliquot was injected. The amount of monacolins was calculated using the calibration curve of monacolin K.

2.5. Method development

The chemical profiling procedure conducted on the liquid chromatography with photodiode array detector was optimized by testing various system conditions. General reverse-phase C_{18} column was used, and several different elution systems were trialed. The resolution and symmetry factors of peaks as monacolin K with its neighboring peaks were dissatisfied by using methanol–water or acetonitrile–water system. The symmetry and resolution was increased by lowering the pH value of elution. Several aqueous solution such as the phosphoric buffer, acetate acid as well as dilute phosphoric acid and dilute trifluoroacetic acid (TFA) together with an organic phase of methanol or acetonitrile etc. were used for the condition optimization. The results suggested a system composed of 0.1% TFA and acetonitrile as an ideal system for the separation of the monacolin compounds. For the consideration of resolution, running time and solvent-saving, the column of Waters Symmetry C_{18} (150 mm × 3.9 mm i.d., 5 μm) was used. The chromatography was performed using a gradient of acetonitrile (eluent A) and 0.1% TFA (eluent B). Linear gradient elution (1 mL/min) from 35 to 75% A in 20 min and keeping 75% A from 20 to 28 min was applied. The total analysis time was 35 min, including column stabilization.

The photo-diode array detector was set at 210–350 nm and the chromatogram detected at 237 nm. The column temperature was set at 30 °C, and the injection volume was 20 μl.

The chromatographic condition of LC/PDA/MS was modified from the condition of LC/PDA. The separations by LC/PDA/MS were performed on a narrow-bore reversed-phase Zorbax SB-C_{18} HPLC column (2.1 mm × 100 mm i.d., 5 μm, Agilent Scientific, CA, USA) with a gradient elution consisting of acetonitrile (eluent A) and aqueous 0.2% acetic acid (eluent B) at a flow rate of 300 μl per minute. The gradient pattern was the same as described for the LC/PDA analysis: linear gradient increasing from 35 to 75% A in 20 min and keeping 75% A from 20

to 28 min. The elution from the HPLC was passed PDA and then directly introduced into the mass spectrometry using eletrospray ionization (ESI). All the analyses were performed using ESI interface with the following settings: positive ionization mode; temperature of the capillary, 250 °C; spray voltage, 4.5 kV; capillary voltage, 6 V; sheath gas (N_2) flow, 30 A.U.; auxiliary gas (N_2) flow, 10 A.U. The electrospray interface and mass spectrometric parameters were optimized to obtain maximum sensitivity unit resolution.

3. Results and discussion

3.1. Chromatographic peaks identification

3.1.1. Identification by LC/PDA

Fermented red rice sample preparations were traced by HPLC/PDA, and chromatograms with on-line UV spectra were shown in Fig. 2. The chromatogram was detected at wavelength of 237 nm and on-line UV spectra from 210 to 350 nm. The peak of monacolin K in samples was identified by comparing to chromatogram, and the UV spectrum obtained with the monacolin K standard, which showed a characteristic mountain-like spectrum with three maximum absorptions at (λ_{max}) 230, 237, and 246 nm, respectively. It was shown that there were 12 other peaks displayed the same or similar UV spectrum of mountain-like peak at λ_{max} 230, 237, and 246 nm as monacolin K gave. The relative retention times (RRT) of these peaks versus monacolin K (1.00) were 0.32, 0.45, 0.50, 0.67, 0.72, 0.78, 0.89, 0.94, 0.97, 1.24, and 1.29, etc.

An alkaline sample was prepared by mixing 4 ml of red yeast rice solution with 1 ml 0.5N NaOH and was applied to the same system of LC/PDA. Comparing the two chromatograms of pre- and post- alkalized red rice solutions revealed that five peaks at RRT 0.45, 0.72, 0.89, 1.00 (MK) and 1.24 disappeared while instead,

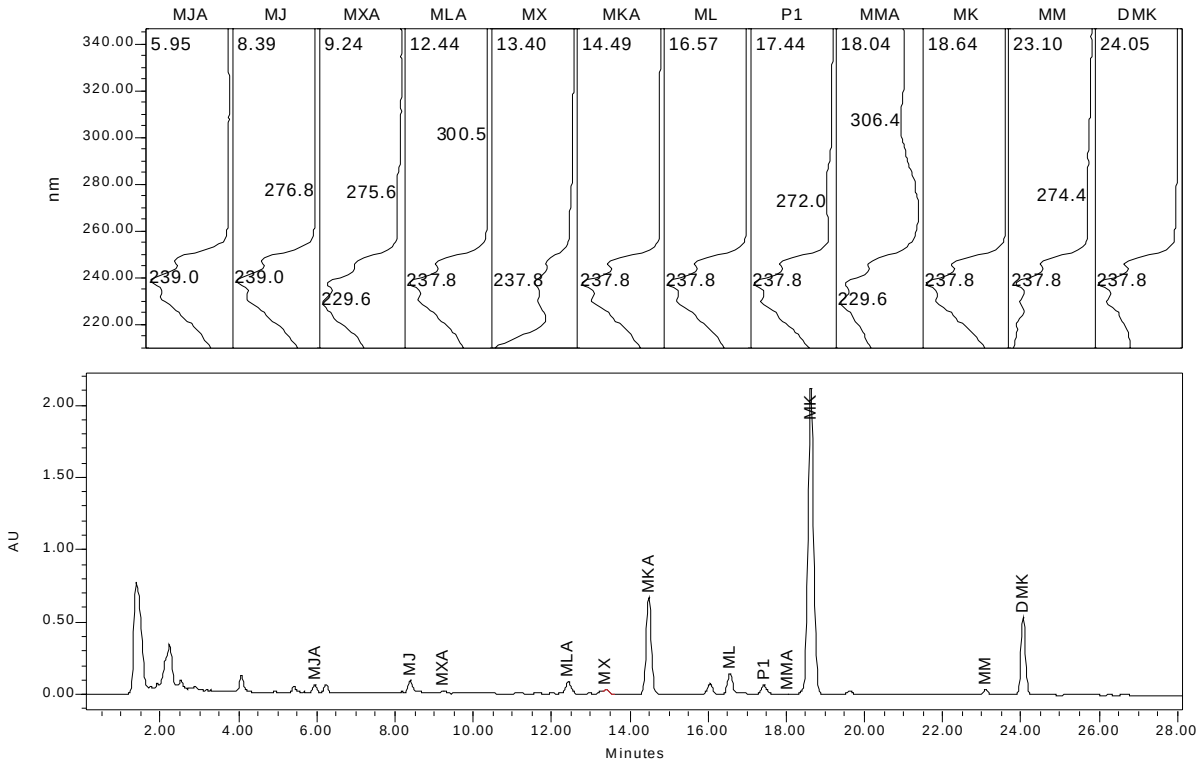


Fig. 2. HPLC profile with UV spectra of the monacolins in fermented red rice.

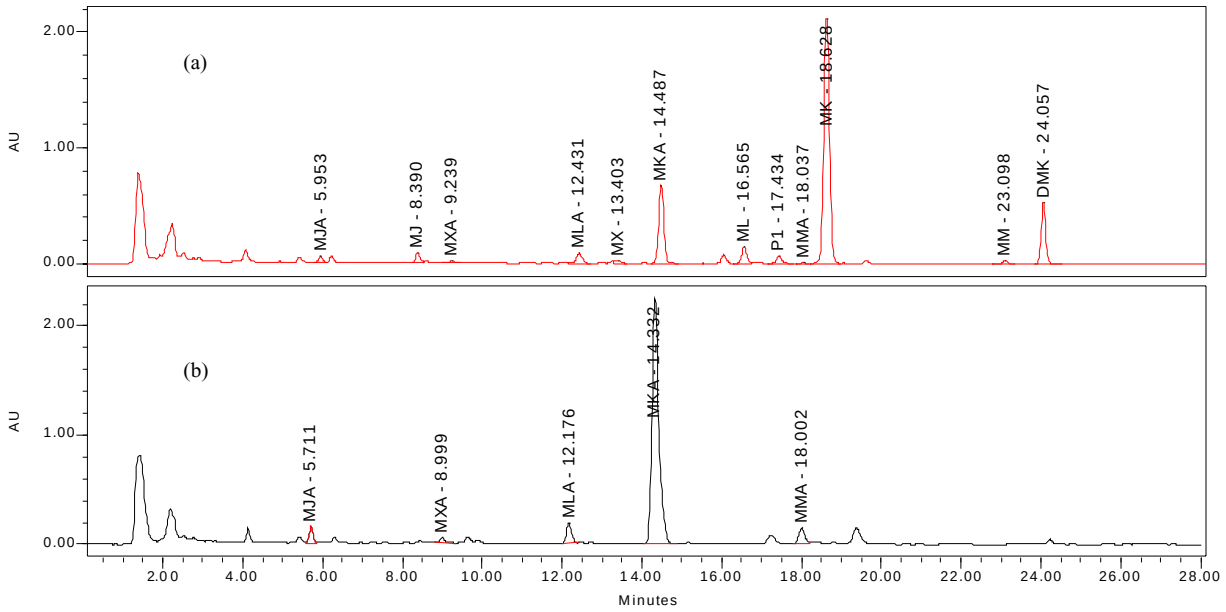


Fig. 3. Comparison of the chromatograms of fermented red yeast rice between pre- and post-alkalization (detected at 237 nm). (a) Chromatogram of pre-alkalization, (b) chromatogram of the post-alkalization.

the peak areas of the other five peaks at RRT 0.32, 0.50, 0.67, 0.78 and 0.97 increased after alkalization (Fig. 3). However, the UV spectra of the missing peaks and those of the area-increasing peaks were of high similarity. A possible explanation was the formation of a hydroxy acid form from the corresponding lactone form upon alkalization (Fig. 4).

3.1.2. Identification by LC/PDA/MS

The red yeast rice preparation was traced by hyphenated instrumentation of liquid chromatography with mass spectrometry, and by subsequence deduction of structural information of the detected molecular ions. Fig. 5 show the total ion current (TIC) chromatogram (a), UV chromatogram (b) and selective ion chromatograms (SIC) (c–p). The molecular ion of the predominant peak at t_R = 16.65 min was 405 (M+1) (c), identified as monacolin K (1, MK), which was confirmed by injection of monacolin K standard. The second strong peak (t_R 13.15 min) displayed the molecular ion 423 ($M+1$) (d) belonging to the hydroxy acid form of monacolin K (1a, MKA). The SIC of 321(t_R 7.09 min) and 339 (t_R 5.22 min) were deduced to be monacolin J (2, MJ) and its hydroxy acid form (2a, MJA), respectively. The SIC of 305 (g) was observed for two peaks : the peak at t_R 14.55 min may be assumed to be monacolin L (3, ML), supported by its characteristic mountain-like UV absorption at λ_{max} 230, 237, 247 nm, and the peak at t_R 6.44 min may be its isomer displaying a UV λ_{max} 260 nm. Similarly, SIC at 323 nm was also observed for two peaks at t_R 10.99 and 4.54 min, and the UV spectrum supported the former (t_R 10.99 min) to be the hydroxy acid form of monacolin L (3a, MLA). Continuously, the SIC at 407 and 425 were contributed by monacolin

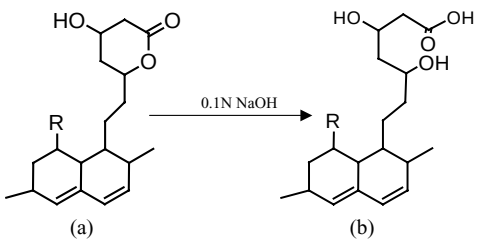


Fig. 4. Transformation of monacolins from lactone form to their hydroxy acid form, (a) monacolins in lactone form, (b) monacolins in hydroxy acid form.

M (5, MM) and its hydroxy acid form (5a, MMA), respectively. Monacolin X (4, MX) and monacolin X hydroxy acid (4a, MXA) were deduced from the SIC at 419 and 437, respectively. Dehydromonacolin K (7, DMK) appeared in SIC at 387, and compactin (6, P1) existed by evidence of the peak of SIC at 391. Other two components, dihydromonacolin L (8, DML) and 3α-hydroxy-3,5-dihydromonacolin L (9, HDML), which showed no peaks in LC chromatogram at 237 nm, still were found in SIC at 307 and 341, respectively. Conclusively, the monacolins (1–5) were found both in lactone and hydroxy acid forms (Fig. 5).

3.2. Chromatographic profiling of monacolins

The chemical profiling of monacolins using liquid chromatography and on-line UV spectrum as shown in Fig. 2 was obtained with the given chromatographic parameters. In this chromatographic profiling, the target peaks were well separated from each other as well as from other unidentified components with the minimum resolution ($R_{s,min}$) of 1.1. The 12 monacolins were identified above by LC/PDA/MS as follows (RRT): MJA(0.32), MJ(0.45), MXA(0.50), MLA(0.67), MX(0.72), MKA(0.78), ML(0.89), P1(0.94), MMA(0.97), MK(1.00), MM(1.24) and DMK(1.29). Five batches of red yeast rice powder and 10 finished products were analyzed based on the established chromatographic profiling (Fig. 6), and the normalized peak area of these monacolins is given in Table 1. The peaks of dihydromonacolin L and 3α-hydroxy-3,5-dihydromonacolin L were too low to be detected due to their trace amount and their low absorption at 237 nm. The data of Table 2 revealed that monacolin K and monacolin K hydroxy acid were the two main components which contributed to up to 90% of the total quantity of monacolins in the red yeast rice powder, and the others shared the remaining 10% only. To demonstrate the similarity or differentiation between the chemical profiling chromatograms between reference and target products, one parameter of

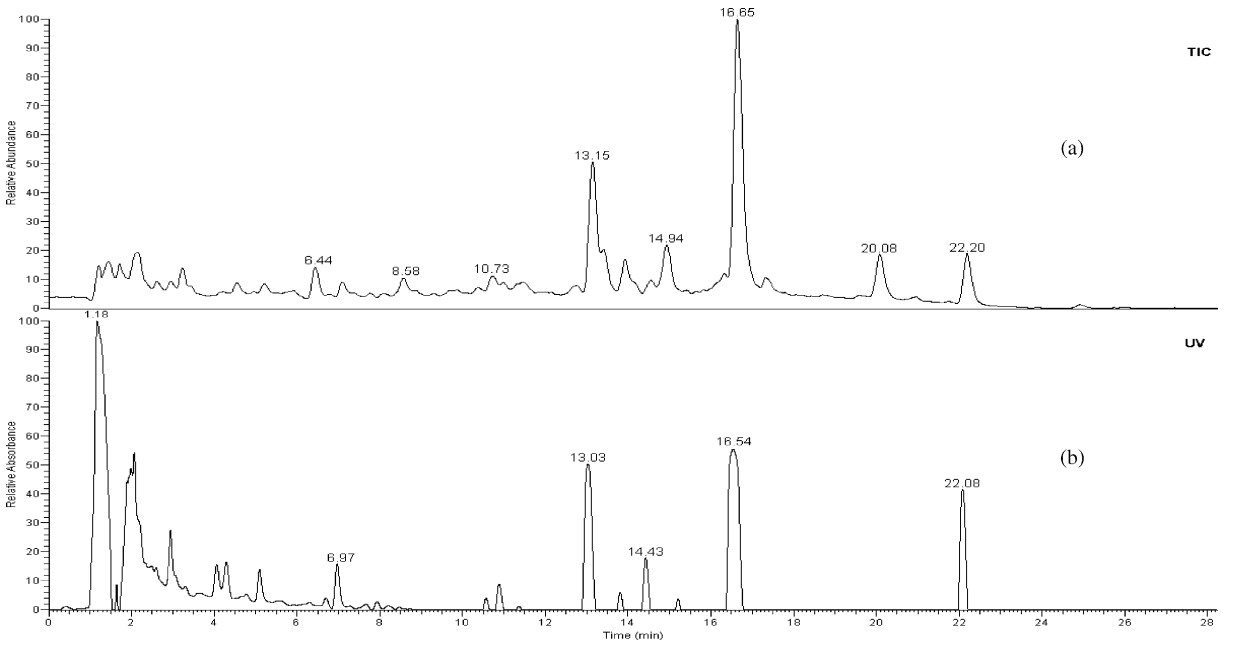


Fig. 5. Chromatograms of red yeast rice and mass spectra of selected peaks (a) total ion current (TIC), (b) HPLC profile at UV237 nm, (c) SIC at M/Z 405, (d) SIC at M/Z 423, (e) SIC at M/Z 321, (f) SIC at M/Z 339, (g) SIC at M/Z 305, (h) SIC at M/Z 323, (i) SIC at M/Z 407, (j) SIC at M/Z 425, (k) SIC at M/Z 419, (l) SIC at M/Z 437, (m) SIC at M/Z 387, (n) SIC at M/Z 390, (o) SIC at M/Z 307, (p) SIC at M/Z 341.

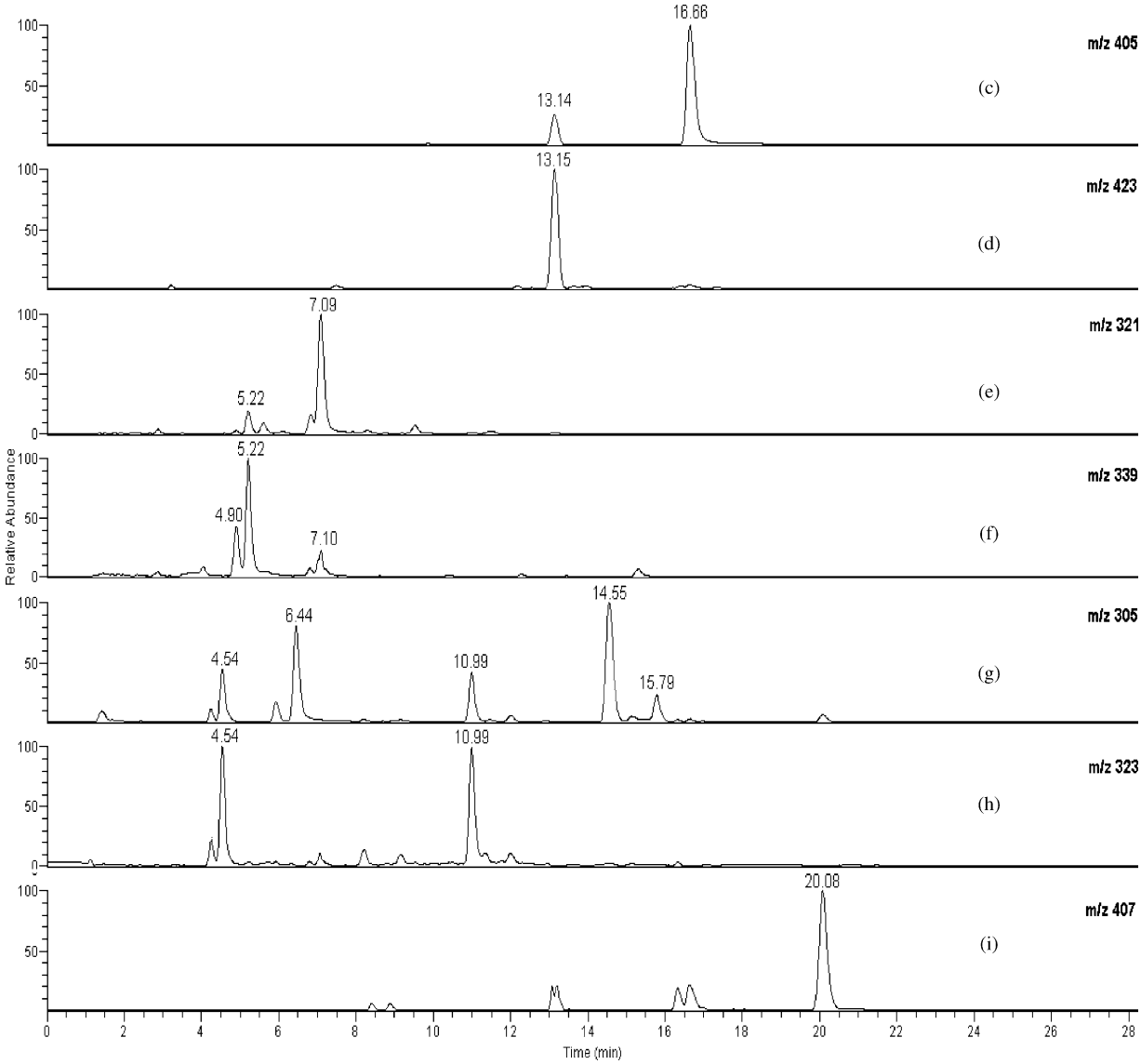


Fig. 5. (*Continued*)

total deviation percentage (TD%) [24] was introduced and calculated by the following equation:

$$\mathrm{TD\%} = \sum_{i}^{n} \frac{|A_s' - A_i'|}{A_s' n}$$

where TD% stands for total deviation percentage, A_s' the normalized peak area of reference chromatogram, A_i' the normalized corresponding peak area of target chromatogram, and n the number of peaks.

The value of TD% demonstrated the similarity between chromatograms of reference and target products. The last column of Table 1 showed that the TD% values of 10 commercial products versus the reference red yeast rice powder. The data of TD% suggested that products CP9 and CP10 were similar to the given

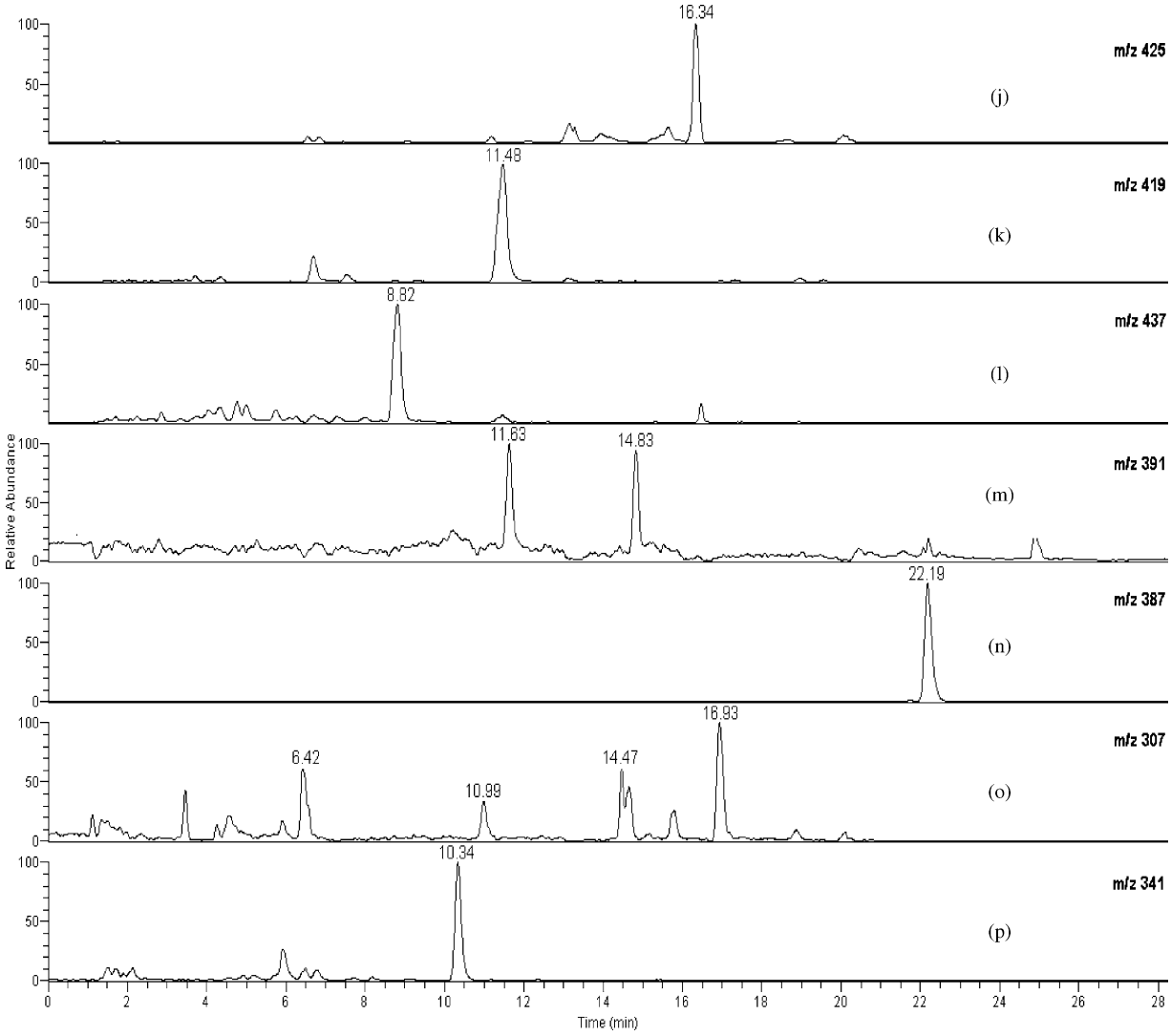


Fig. 5. (*Continued*).

reference, but the others significantly differ from the reference powder.

3.3. Quantitation of red yeast rice and related products

The contents of 12 monacolins in one red yeast rice powder and ten commercial red yeast rice products were determined by HPLC/PDA. All the monacolins were calculated with monacolin K as the reference standard. The results (Table 2) showed that the contents of monacolins in these products were considerably different, not only in individual components but also in the total quantity of monacolins.

3.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The diluted sample solutions were injected for 10 μl. The concentration of 0.5 μg/ml resulted in the peak height of monacolin K approximately three times higher than baseline noise as LOD while 1.5 μg/ml resulted in approximately 10 times higher than baseline noise as LOQ for each of the ingredients. At

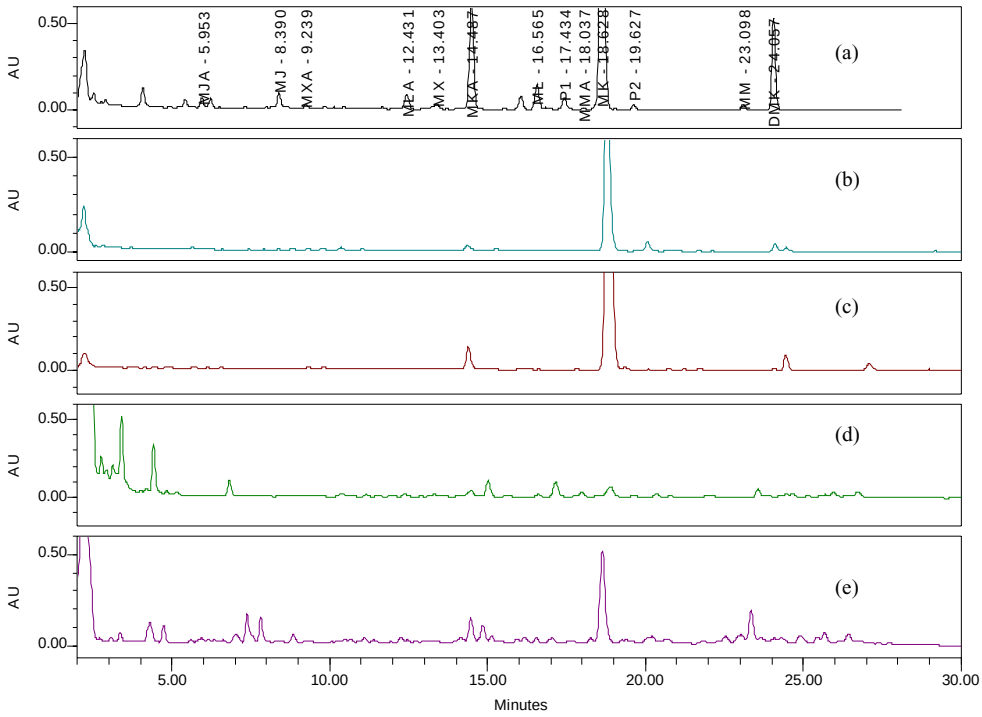


Fig. 6. Chromatographic chemical profiling of monacolins in fermented red yeast rice and commercial products (a) fermented red yeast rice powder, (b–e) commercial products, (b)CP7, (c) CP3, (d) CP4, (e) CP5.

Table 1
Data of chemical profiling of the monacolins in red yeast rice and commercial products (peak area in percentage)

Batch	MJA	MJ	MXA	MLA	MX	MKA	ML	P1	MMA	MK	MM	DMK	TD%
RRT	0.319	0.45	0.5	0.667	0.719	0.778	0.889	0.936	0.968	1	1.24	1.291	
R1	1.11	1.98	0.57	2.33	0.79	17.13	3.64	1.91	0.12	57.65	0.81	11.96	
R2	1.08	1.97	0.59	2.42	0.74	18.25	3.55	1.86	0.11	56.54	0.82	12.07	
R3	1.11	1.98	0.62	2.37	0.79	16.05	3.68	1.99	0.14	57.86	0.84	12.57	
R4	1.05	1.95	0.57	2.4	0.74	17.58	3.95	2.17	0.11	57.16	0.79	11.53	
R5	1.16	1.94	0.51	2.46	0.71	22.13	3.65	1.52	0.12	53.65	0.82	11.33	
Avg.	1.102	1.964	0.572	2.396	0.754	18.228	3.694	1.89	0.12	56.572	0.816	11.892	
SD	0.04	0.02	0.04	0.05	0.04	2.32	0.15	0.24	0.01	1.71	0.02	0.49	
CP1	–	–	–	–	–	1.16	–	–	–	98.51	–	0.33	89.61
CP2	–	–	–	–	–	2.87	–	–	–	95.35	–	1.78	87.52
CP3	–	–	–	–	–	4.83	–	–	–	79.04	–	16.13	80.68
CP4	–	–	–	–	–	2.53	–	–	–	93.32	–	4.15	85.86
CP5	–	–	–	–	–	39.08	–	–	–	60.92	–	–	86.31
CP6	7.96	–	–	–	–	92.04	–	–	–	–	–	–	155.94
CP7	–	–	–	–	–	12.39	2.78	1.1	–	73.77	0.57	9.39	60.01
CP8	–	–	–	–	–	3.85	–	–	–	93.95		2.2	86.65
CP9	1.15	1.92	0.55	2.52	0.78	19.02	3.76	1.81	0.15	57.75	0.73	9.86	6.47
CP10	0.78	2.02	0.6	2.37	0.72	18.33	3.82	1.92	0.13	55.34	0.72	13.25	6.29

– Not detectable. MJA, monacolins J hydroxy acid; MJ, monacolin J; MXA, monacolin X hydroxy acid; MLA, monacolin L hydroxy acid; MX, monacolin X; MKA, monacolin K hydroxyl acid; ML, monacolin L; P1, compactin; MMA, monacolin M hydroxy acid; MK, monacolin K; MM, monacolin M; DMK, dehydroxymonacolin K, etc. R1–R5, red yeast rice powder 1–5; CP1-CP10, commercial products 1–10.

Table 2
Quantification of monacolins in red yeast rice and commercial products

Batch		MJA	MJ	MXA	MLA	MX	MKA	ML	P1	MMA	MK	MM	DMK	total
Ref (powder)	μg/g	7.08	12.64	3.64	15.36	4.74	103.23	23.32	12.14	0.72	362.37	5.19	72.22	622.65
CP1	μg/capsule	–	–	–	–	–	3.57	–	–	–	302.48	–	1.03	307.08
Cp2	μg/capsule	–	–	–	–	–	2.81	–	–	–	93.65	–	1.74	98.2
Cp3	μg/capsule	–	–	–	–	–	6.9	–	–	–	112.76	–	22.68	142.34
CP4	μg/capsule	–	–	–	–	–	3.44	–	–	–	126.61	–	5.63	135.68
CP5	μg/tablet	–	–	–	–	–	6.75	–	–	–	10.52	–	–	17.27
CP6	μg/tablet	1.11	–	–	–	–	12.8	–	–	–	–	–	–	13.91
CP7	μg/tablet	–	–	–	–	–	19.60	4.48	1.75	–	112.00	0.91	15.05	155.68
CP8	μg/capsule	–	–	–	–		11.55	–	–	–	3.4.19	–	7.14	18.69
CP9	μg/capsule	4.33	7.22	2.08	8.86	2.75	63.48	13.47	6.97	0.44	198.65	3.01	46.5	357.76
CP10	μg/tablet	5.62	8.56	3.12	9.23	2.94	82.66	18.46	8.23	0.52	259.32	3.22	59.68	461.56

–, Not detectable. MJA, monacolins J hydroxy acid; MJ, monacolin J; MXA, monacolin X hydroxy acid; MLA, monacolin L hydroxy acid; MX, monacolin X; MKA, monacolin K hydroxy acid; ML, monacolin L; P1, compactin; MMA, monacolin M hydroxy acid; MK, monacolin K; MM, monacolin M; DMK, dehydroxymonacolin K, etc. Ref (powder), red yeast rice powder; CP1–CP10, commercial products 1–10.

the quantitation limit of 1.5 μg/ml, the data of six repeated injections indicated repeatability within a deviation of 6–7% (RSD).

4. Conclusion

Monacolins were a class of compounds produced during fermentation, of which only a part of the analogs have been identified in this report. The fermentation condition might directly effect the production of these secondary metabolites. The combination of LC/PDA and LC/PDA/MS has become a powerful tool to identify even trace amount of secondary metabolites like monacolins, which, as a series of derivatives, have the same skeleton and display similar UV spectra. The substitution on the functional groups and the lactone or hydroxy acid form provide important information of molecular and daughter ions, which could be detected and traced by tandem mass spectra. Chromatographic chemical profiling proved to be an effective and convenient approach for the qualitative evaluation of this kind of products as it yielded detailed chemical information involving both the marker and related compounds.

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