FOOD CHEMICAL CONTAMINANTS

Synchronous Analysis Method for Detection of Citrinin and the Lactone and Acid Forms of Monacolin K in Red Mold Rice

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The *Monascus* product known as red mold rice (RMR) has been found to contain the cholesterol-lowering agent monacolin K (MK), including the lactone form (MKL) and the acid form (MKA) and mycotoxin citrinin (CT). In current studies, CT and MK are usually detected by different analysis methods, which have a high level of error, and are inconvenient, expensive, and time-consuming. The goal of this study is to establish a rapid synchronous analysis method for the detection of CT, MKL, and MKA levels in RMR. In this study, CT, MKL, and MKA are extracted by the same extraction method and are then separated in a reversed-phase high-performance liquid chromatography (HPLC) C_{18} column. The elution from the C_{18} column is then passed through an ultraviolet detector and introduced directly into the fluorescence detector. The results show that higher recovery rates of CT, MKL, and MLK are yielded from RMR powder by extracting with 95% ethanol (10 mL) at 60°C for 30 min. Regarding the optimal conditions of HPLC, the peaks of CT, MKL, and MKA can be clearly separated from any noise peaks by isocratic elution with optimum mobile phase, acetonitrile–water–trifluoroacetate (55 + 45 + 0.05, v/v).

Because the *Monascus* species contains multifunctional compounds, it is receiving much attention in the field of functional foods research. In many countries, cardiovascular disease has recently become one of the most serious problems. Prophylaxis and therapy are therefore becoming increasingly important research subjects. Monacolin K (MK, also known as lovastatin, mevinolin, and mevacor) is a potent and competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis (1, 2). It not only inhibits cholesterol biosynthesis, but also lowers blood cholesterol level in both human and animals.

Red mold rice (RMR), a Chinese traditional food, has been used as a dietary supplement and as a remedy in China and Japan for thousands of years. RMR is still restricted and not accepted in many countries, and causes much controversy, because mycotoxin citrinin (CT) is secreted by *Monascus* spp. This fact, proven by Blanc et al. (3), indicates that *Monascus purpureus* and *M. ruber* produce an antibacterial compound, monascidin, which is the same compound as CT (3). CT is a potent renal toxin and hepatotoxin, which causes functional and structural kidney damage as well as alterations in liver metabolism (4). It inhibits several enzymes linked to the respiratory chain of the kidney cortex and liver mitochondria, as well as malate and glutamate dehydrogenases and the adenosine triphosphate (ATP)-synthetase complex (4). CT is often found in solid and submerged cultured products of *Monascus* spp. and is detected from 0.2 to 122 mg/kg in RMR (3, 5).

Recently, functional food related to *Monascus* is being regarded as the cholesterol-lowering agent MK. Accordingly, MK levels in the *Monascus* product are associated with cholesterol-lowering effect in vivo, impacting the receptivity of the *Monascus* product. At the same time, in current studies, CT has been found to be a serious problem in many *Monascus* products (4–6). For these reasons, commercial *Monascus* products are of a serious concern to the public because of their CT and MK levels, and the quantitative analysis of MK and CT becomes an important subject in *Monascus* research. In determining of secondary metabolites of *Monascus*, many researchers focus on creating a synchronous analysis system for various forms of monacolin or pigment (7–9). The methods of CT detection reported in current studies include thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), and

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enzyme-linked immunosorbent assay (ELISA; 3, 10–13). HPLC is regarded as a suitable, sensitive, and universal method for CT detection. However, several CT analysis methods in current studies depend on the mass spectra or gradient elution mode (3, 14). LC/mass spectrometry (LC/MS) carries a higher instrument cost, and the gradient elution mode usually leads to an instable baseline, interfering with the integration of selected peaks in the HPLC chromatogram. Therefore, a universal and simple method for CT detection needs to be developed. In addition, because the MK lactone (MKL) and acid forms (MKA) are both beneficial for lowering cholesterol levels in vivo, the MK concentration of RMR should include these 2 MK forms. For these reasons, the detection of CT, MKL, and MKA in a *Monascus* product has become more important.

In the present study, CT and MK are detected by different analysis methods, which result in a lengthy analysis that is costly, inconvenient, and has a high level of error. The purpose of this study is to establish a universal, sensitive, and rapid synchronous analysis method for determining the CT, MKL, and MKA levels of RMR. CT can present a sensitive fluorescent emission and form a great peak area in HPLC chromatograms under fluorescent conditions. Furthermore, MKA and MKL have identical spectra and a maximum absorption at the ultraviolet (UV) wavelength of 238 nm. This study describes a synchronous analysis system for determining the CT, MKL, and MKA levels of RMR by using the same C_{18} reversed chromatographic column and by connecting the fluorescence detector with the UV detector.

Experimental

Materials

(**a**) *Standard*.—MK (mevinolin) and citrinin were purchased from Sigma Chemical Co. (St. Louis, MO). In general, MK can be divided into 2 forms: MKL and MKA. The commercial standard of MK (MKL) can be transformed to MKA by alkalization. The preparation of the MKA standard solution was as follows: MKL was dissolved in a 0.1 N sodium hydroxide methanol solution in a concentration range of 100–1000 mg/L. Standard solutions of CT and MKL were prepared by dissolving CT and MKL in 100% methanol in a concentration range of 100–500 μg/kg and 100–1000 mg/kg, respectively.

(**b**) *Chemicals*.—Acidifier including trifluoroacetate (TFA), acetic acid, and phosphoric acid were purchased from Sigma Chemical Co. LC grade acetonitrile, reagent grade ethyl acetate, methanol, acetone, chloroform, sulfuric acid, and isooctane were purchased from Merck Co. (Darmstadt, Germany).

(**c**) *Monascus-related food*.—RMR, *Monascus* capsule, and *Monascus* processed food were purchased from markets in Taipei, Taiwan.

Apparatus

(**a**) *HPLC synchronous analysis system*.—A synchronous analysis system of CT, MKL, and MKA consisted of injector, eluent pump, C_{18} column, UV detector, and fluorescence detector. In order to detect CT, MKL, and MKA with the same test, this system was connected to the fluorescence detector via the UV detector. In other words, the eluent from the C_{18} column was passed through the UV detector and then directly introduced into the fluorescence detector. CT was detected by fluorescence detector, and the excitation and emission wavelengths were set at 330 and 500 nm, respectively (15). Both MKA and MKL were detected by UV detector set at 238 nm (16).

(*1*) *Column*.—C_{18}, 25 cm × 4.6 mm id, 5 μm (Discovery@, Supelco, Inc., Bellefonte, PA). (*2*) *Injector*.—7725i (Rheodyne Co., Rohnert Park, CA). (*3*) *Eluent pump*.—PU2089 plus (Jasco Co., Tokyo, Japan). (*4*) *UV detector*.—UV2075 plus (Jasco Co.). (*5*) *Fluorescence detector*.—FL-1 (Rainin Co., Wobum, MA).

(**b**) *LC/MS system*.—LC system (Finnigan MAT Spectra System, San Jose, CA), including quaternary pump, online degasser, column heater compartment, autosampler. LCQ mass spectrometer (Finnigan MAT LCQ) consisting of an electrospray ionization (ESI) interface and ion trap mass analyzer. The software controlling the equipment and treatment of data was obtained from Xcalibur Core Data System Software Revision 1.0 (Xcalibur Software Inc., Herndon, VA).

HPLC Conditions of Synchronous Analysis System

CT, MKA, and MKL were determined by HPLC on a C_{18} column using the gradient elution or isocratic elution. The mobile phase of the gradient elution as well as the isocratic elution was a composite of acetonitrile, water, and acidifier (0.05%). Phosphoric acid was chosen as the acidifier of the mobile phase for the original HPLC condition (16). Except for phosphoric acid, 0.5 mL TFA or acetic acid was added to 1000 mL of the mobile phase in order to find an optimal acidifier for stimulating the peak area of CT, MKL, and MKA.

(**a**) *Linear gradient elution*.—The method of linear gradient elution was as follows: acetonitrile–water (20 + 80, v/v) to acetonitrile–water (80 + 20, v/v) in 30 min, and then an isocratic elution acetonitrile–water (80 + 20, v/v) from 30 to 40 min. The eluent was then pumped at a flow rate of 0.8 mL/min.

(**b**) *Isocratic elution*.—CT, MKL, and MKA were separated by isocratic elution using the mobile phase with the composition of acetonitrile–water–acidifier (55 + 45 + 0.05, v/v). The flow rate was set at 1.0 mL/min.

LC/MS Conditions

The chromatographic condition of LC/MS was modified from that of HPLC. The separation by LC/MS was performed by a reversed-phase C_{18} HPLC column with an isocratic elution at a flow rate of 0.5 mL/min. The composition of the mobile phase was prepared as described for the optimal condition of the HPLC analysis. All the analyses were performed using the ESI interface with the following settings: positive ionization mode; temperature of the capillary, 25°C; spray voltage, 4.5 kV; capillary voltage, 6 V; sheath gas (N_2)

flow, 30 AU; auxiliary gas (N_2) flow, 10 AU. The electrospray interface and MS parameters were optimized to obtain maximum sensitivity unit resolution.

Published Individual Analysis Method

(**a**) *Detection of CT*.—CT was extracted from RMR (1 g) with 10 mL methanol at 50°C for 1.5 h. The filtered extract was defatted twice with isooctane. After adding an equal volume of water and acidifying to pH 4.5 with H_2SO_4 (50 + 50, v/v), the extract was partitioned with $CHCl_3$. The lower phase was evaporated to dryness and then dissolved in methanol, followed by filtering with 0.45 μm pore size filter and analysis by HPLC (3). CT was determined by HPLC on a C_{18} column using the mobile phase with the composition of acetonitrile–water–trifluoroacetate (55 + 45 + 0.05, v/v). The flow rate was set at 1.0 mL/min and a fluorescence detector was used. The excitation and emission wavelengths were set at 330 and 500 nm, respectively (15).

(**b**) *Detection of MK*.—MKL and MKA were extracted from RMR powder (1 g) with 5 mL ethyl acetate at 70°C for 1.5 h. The suspension was then filtered through filter paper. The filtrate was evaporated to dryness under vacuum. A 1 mL volume of acetonitrile was added to the resulting mixture, followed by filtering through 0.45 μm pore size filter and analysis by HPLC (5). Chromatographic separation was conducted on a C_{18} column. Mobile phase of 0.5% acetonitrile–phosphoric acid (65 + 35, v/v) was used. The eluent was pumped at a flow rate of 0.7 mL/min. UV detection was set at 238 nm (5).

Extraction Experiment

The basic process of extraction was as follows: RMR powder (1 g) was extracted with 5 mL extraction solvent, under unshaken condition, at 70°C for 1.5 h (5). Various kinds of solvent (methanol, ethanol, acetonitrile, ethyl acetate, and acetone), at various extraction temperatures (50, 60, 70, and 80°C), at various extraction times (30, 60, 90, and 120 min), under shaken or unshaken extraction, were used to investigate the optimal extraction condition. After extraction, a 1 mL suspension was transferred to a new Eppendorf tube and then evaporated to dryness in the glass desiccator under vacuum conditions. A 1 mL volume of acetonitrile was added to the resulting mixture, followed by filtration with a 0.45 μm pore size filter, and analyzed by HPLC.

Extraction of Liquid Samples

MK and CT dissolve better in organic solutions such as chloroform than in water (17). This principle was applied to the extraction process of MK and CT of liquid samples. The extraction protocol was as follows: A 1 mL volume of liquid sample was extracted with 5 mL chloroform under shaken conditions (100 rpm) at 37°C for 30 min. After extraction, 5 mL organic suspension was taken and then evaporated to dryness under vacuum. A 0.5 mL volume of acetonitrile was added to the resulting mixture, followed by filtration with a 0.45 μm pore size filter and analysis by HPLC.

Recovery Experiment

The character of RMR fermented by *Monascus* spp. should differ from that of unfermented rice. For this reason, RMR with known CT (125 μg/kg), MKL (242 mg/kg), and MKA (54 mg/kg) levels were chosen as the control samples for the recovery experiment. Various concentrations of MKA (100, 200, 300, 400, or 500 mg/kg); MKL (100, 200, 300, 400, or 500 mg/kg); and CT (50, 100, 150, 200, or 250 μg/kg) were added to the same test RMR sample powder (1 g), respectively, and then evaporated to dryness under vacuum. The test RMR powder sample with extra CT, MKL, and MKA was detected by using the optimal condition of the synchronous analysis method. In addition, CT, MKL, and MKA levels were detected in the control RMR. The calculation of the recovery rate was as follows:

$$\text{Recovery rate, \%} = (A' - B)/(A) \times 100\%$$

where A′ = total levels of the target compound of the test RMR sample detected by the synchronous analysis method; A = levels of the added target compound; B = original levels of the target compound of the control RMR detected by the synchronous analysis method (CT, 125 μg/kg; MKL, 242 mg/kg; and MKA, 54 mg/kg).

Comparison of Synchronous Analysis Method and Published Individual Analysis Method

RMR powder with extra known CT, MKL, and MKA levels were prepared as described in the recovery experiment. The synchronous analysis method followed the optimal extraction and HPLC analysis conditions used in this study. The accuracy of analysis method was evaluated according to R^2 and CM (the ratio of citrinin to MK) values. R^2 value was calculated by statistic analysis software of Microsoft Excel 2002. CM value, regarded as a ratio of CT levels to total MK levels in the RMR, was modulated to 0.5 by adding known concentrations of CT, MKL, and MKA to RMR powder. The error of analysis method decreased as the CM value gained by detection of RMR became closer to 0.5. The calculation of the CM value was as follows:

$$\text{CM value} = A \times 1000/(B + C)$$

where A = the detected levels of CT (μg/kg); B = the detected levels of MKL (mg/kg); C = the detected levels of MKA (mg/kg).

Results and Discussion

Separation of CT, MKA, and MKL by Gradient and Isocratic Elution

The *Monascus* metabolite includes a large amount of pigment as well as CT and MK. They all are members of a polyketide derivative (18–22), and for this reason their structure and character are similar to each other. Previous studies have indicated that the involvement of pigment interferes with the separation and purification of CT (14). The purpose of this study is to establish a rapid and simultaneous

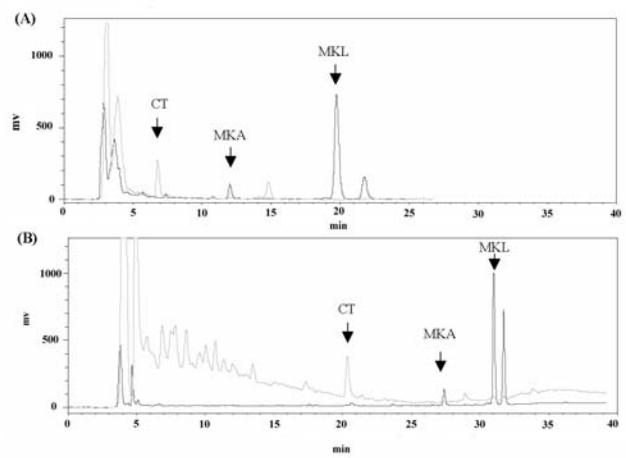

Figure 1. Overlap high-performance liquid chromatograms for the UV spectra and fluorescence spectra under isocratic elution (A) and gradient elution (B). CT = Citrinin; MKL = lactone form of monacolin K; MKA = acid form of monacolin K.

method for detecting CT, MKL, and MKA of RMR. Therefore, the quantitative analysis method should be suitable for these compounds.

In this study, gradient elution and isocratic elution were used, respectively, as the HPLC condition in order to investigate the effect of separation between selected peaks and noise. As shown in Figure 1, the 2 elution modes presented a good result for separating the selected peaks from noise, but gradient elution would lead to an unstable baseline and further interfere with the integration of the CT peak area in the fluorescence chromatogram. CT could be separated from a mound of noise peaks by isocratic elution, as well as by MKA and MKL. Based on the results, isocratic elution was chosen as the HPLC condition for simultaneous detection of CT, MKL, and MKA.

The Effect on HPLC of Various Acidifiers in the Mobile Phase

In the current study, HPLC is usually recommended as a general and convenient method for the quantitative determination of CT, MKL, and MKA (5, 7, 8, 11, 14). Many researchers have suggested that CT and MK should be detected under an acidic condition (7, 11, 23). When CT is situated at a pH value < 4.6, the carbonyl group will bind to the benzene and ketone groups by hydrogen bond and instantly form a pseudo-pentaheterocyclic structure. CT can present the maximum fluorescent emissivity at an emission wavelength of 500 nm if the pH value is acidified to 2.5, while at the same time CT is closed to hydrophobic character. In contrast, adjusting the pH value to >4.6 leads to a lower fluorescent emissivity and hydrophilic character in CT (11). Furthermore, MKA and MKL have identical spectra and need to be placed under an acidic condition in order to provide maximum absorption at 238 nm. It therefore follows that selecting a suitable acidifier for forming the maximum peak area in high-performance liquid chromatograms is very important.

In this study, solvent extract of RMR was eluted through C_{18} column in the mobile phase with various acidifiers (phosphoric acid, acetic acid, and TFA). Figure 2 A–C show that using TFA as the acidifier stimulates MKA and MKL to form higher and conspicuous peaks in the HPLC chromatograms at the UV wavelength of 238 nm. The influence on the CT peak by the acidifier is shown in Figures 2 D–F. Acetic acid, which was chosen as the acidifier of the mobile phase, displayed a poor effect on the fluorescent emissivity of CT, while phosphate and TFA rendered CT to express a strong fluorescent emissivity and a sharp peak in the high-performance liquid chromatograms. As indicated above, we can conclude that selecting TFA as the acidifier of the mobile phase is beneficial to increase the peak area of CT, MKL, and MKA in high-performance liquid chromatograms.

The Effect on HPLC of Various Ratios of Mobile Phase

The ratio of acetonitrile to distilled water influences the separation effect of HPLC between selected peaks and noise peaks. In order to investigate the optimal condition for separating the peaks of CT, MKL, and MKA, various ratios of aqueous acetonitrile at 50, 55, and 60% were used as the mobile phase, respectively. As shown in Figure 3, fluorescence and UV chromatograms of the same sample overlap the high-performance liquid chromatogram. There were good separation effects on CT, MKL, and MKA peaks when 55% aqueous acetonitrile was used as the mobile phase. According to the foregoing results, the mobile phase with

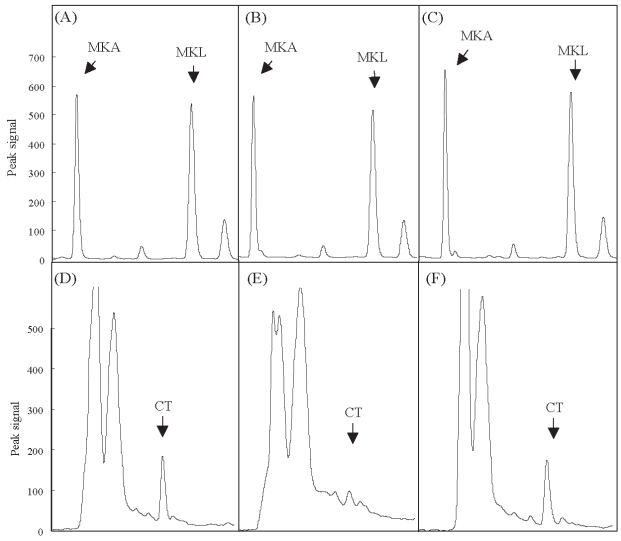

Figure 2. High-performance liquid chromatograms for UV spectra (A–C) and fluorescence spectra (D–F) with various acidifiers of mobile phase. (A, D) Phosphoric acid, (B, E) acetic acid, (C, F) trifluoroacetate. CT = Citrinin; MKL = lactone form of monacolin K; MKA = acid form of monacolin K.

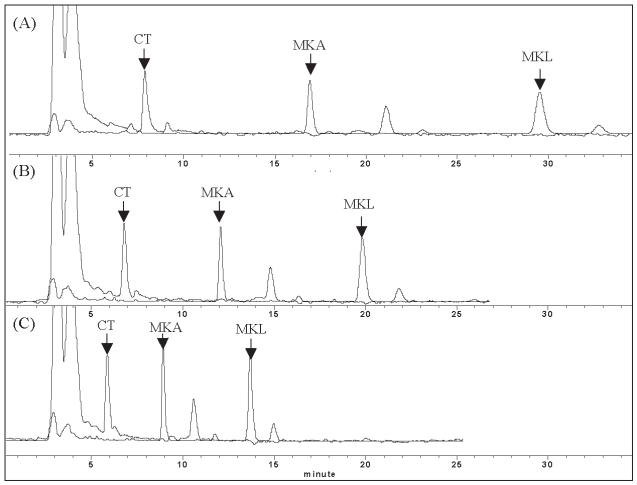

Figure 3. Comparison of the chromatograms of RMR extract among various ratios of mobile phase. (A) Acetonitrile–water (50:50), (B) acetonitrile–water (55:45), (C) acetonitrile–water (60:40). CT = Citrinin; MKL = lactone form of monacolin K; MKA = acid form of monacolin K.

acetonitrile, water, and TFA (55 + 45 + 0.05) was selected as the optimal condition in the HPLC analysis. The standard curves of CT, MKA, and MKL were then further investigated. HPLC connected with a fluorescence detector performed a sensitive detection in the CT levels, registering the detectable limit at 10 μg/kg. R^2 values calculated from the standard curve of CT, MKA, and MKL were 0.9978, 0.9981, and 0.9914, respectively (data not shown).

Extraction Solvent

The investigation of the extraction effect was an important part of this study. It has been reported that both CT and MK dissolve in many organic solvents such as methanol and acetonitrile. Several organic solvents suitable for dissolving CT and MK were used to investigate the extraction effect for RMR. Figure 4 shows that methanol and ethanol used as the extraction solvent had better extraction levels in MKA and MKL than other extraction solvents. The extraction levels of CT demonstrated significant variation among various solvents, especially acetone and ethyl acetate, which had lower CT levels from RMR than did the other solvents. Among the solvents, ethanol extracted the highest amount of CT levels from RMR and was therefore chosen as the solvent for extracting CT, MKA, and MKL.

Extraction Temperature

Heating is usually used to accelerate the breakage of the cells and obtain a higher level of extraction, especially for an intracellular sample. Therefore, RMR was immersed in ethanol and heated at 50, 60, 70 and 80°C for 90 min. As shown in Figure 5, heating improved the extraction levels for MKL and MKA. However, the extraction effect did not increase when the temperature rose above 70°C. In addition, the extraction levels of MK had a tendency to decrease at 80°C. This might be due to pyrolysis of the MK structure. At high temperature, a similar effect was observed for CT. The extraction temperature was therefore set at 60°C because the extraction levels of CT, MKL, and MKA were best at this temperature.

Extraction Time and Shaking Extraction

It is anticipated that extraction will obtain the largest amount of target compound in the shortest time, thereby reducing both time and cost. Figure 6 shows an ambiguous effect on the extraction levels over an extended period of time. However, the maximum MKA, MKL, and CT levels were extracted from RMR in 30 min. Extending the extraction time did not increase the extraction levels of the target compounds, but surprisingly actually resulted in the decrease of CT levels. A comparison between unshaken and shaken extractions for MKA, MKL, and CT levels is shown in Figure 6. Shaken extraction with a rotation rate of 130 rpm did not increase but instead decreased the extraction levels of the target compound such as CT, as compared with the unshaken extraction. In order to establish a simple and convenient analysis method, the RMR was immersed in ethanol and extracted by the unshaken extraction method at 60°C for 30 min.

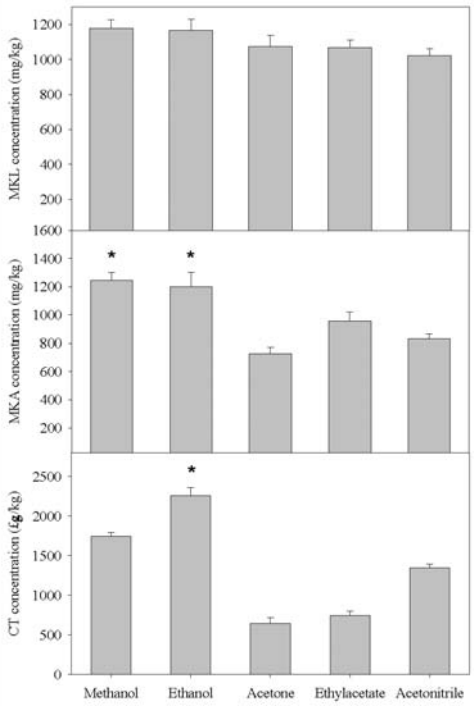

Figure 4. Effect on the extraction levels of lactone form of monacolin K (MKL), acid form of monacolin K (MKA), and citrinin (CT) by various extraction solvents. Each data point of 3 experiments was measured in triplicate, and values represent the mean ± standard error. * = Significant differences between groups ($P < 0.05$).

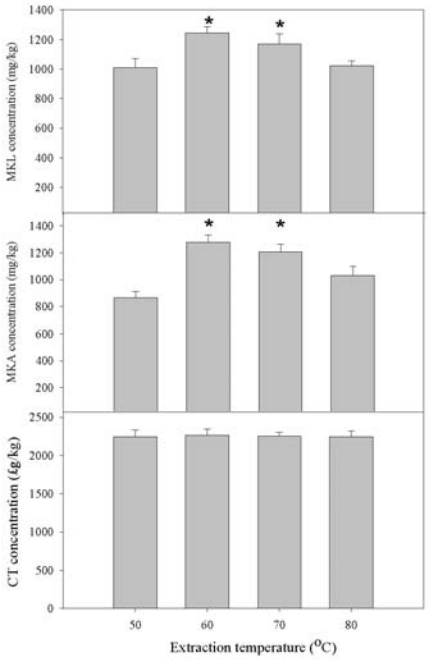

Figure 5. Effect on the extraction levels of lactone form of monacolin K (MKL), acid form of monacolin K (MKA), and citrinin (CT) by various extraction temperatures. Each data point of 3 experiments was measured in triplicate, and values represent the mean ± standard error. * = Significant differences between groups ($P < 0.05$).

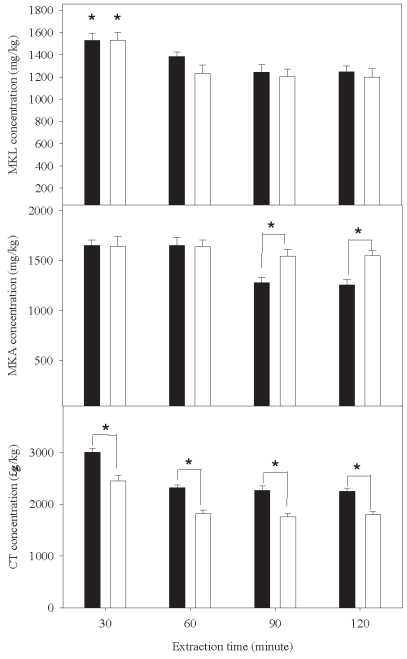

Figure 6. Comparison of the extraction levels of lactone form of monacolin K (MKL), acid form of monacolin K (MKA), and citrinin (CT) between unshaken (■) and shaken (□) extraction by various extraction temperatures. Each data point of 3 experiments was measured in triplicate, and values represent the mean ± standard error. * = Significant differences between groups ($P < 0.05$).

Recovery Experiment

In order to investigate the recovery rate in the extraction process, standard solutions of CT, MKL, and MKA or distilled water were added to RMR powder with known concentrations of CT, MKL, and MKA to be regarded as the test or control RMR sample. After evaporating to dryness under vacuum, test RMR and control RMR samples were both extracted and detected by the quantitative analysis method established in this study. The control RMR sample was regarded as a starting material without extra CT, MKL, and MKA after subtracting the original CT, MKL, and MKA concentrations in the control RMR. The extra CT, MKL, and MKA concentrations of the test RMR sample were obtained by subtracting the original concentration from the total examined concentration. Table 1 shows that the extraction method established in this study resulted in a recovery rate of 94.0 and 91.8% in MKL and MKA, respectively. The recovery rate in CT extraction unfortunately had a lower level of 86.2%.

In an additional experiment, the standard CT was added to unfermented rice powder before heating the extraction process and HPLC analysis. The result unexpectedly

Table 1. Recovery rate for the determination of CT, MKL, and MKA of RMR

Amount added			Recovery rate, %		
Citrinin, μg/L	Monacolin K lactone form, mg/L	Monacolin K acid form, mg/L	Citrinin	Monacolin K lactone form	Monacolin K acid form
50	100	100	83.2 ± 4.23	93.1 ± 3.54	90.3 ± 5.86
100	200	200	85.4 ± 4.76	94.5 ± 4.39	93.4 ± 6.47
150	300	300	88.5 ± 5.21	93.2 ± 5.32	92.2 ± 7.54
200	400	400	87.4 ± 3.26	95.0 ± 5.92	92.3 ± 6.12
250	500	500	86.3 ± 5.91	94.4 ± 5.01	91.0 ± 4.20
Average			86.2	94.0	91.8

Table 2. Comparison of synchronous analysis method and traditional individual analysis method for CT, MKL, and MKA of RMR

Amount added			Synchronous analysis method				Traditional individual analysis method			
Citrinin, mg/kg	Monacolin K lactone form, mg/kg	Monacolin K acid form, mg/kg	Citrinin, mg/kg	Monacolin K lactone form, mg/kg	Monacolin K acid form, mg/kg	CM value[a]	Citrinin, mg/kg	Monacolin K lactone form, mg/kg	Monacolin K acid form, mg/kg	CM value[a]
0.1	100	100	0.087	97	94	0.453	0.076	95	92	0.409
0.2	200	200	0.178	198	195	0.452	0.137	200	191	0.350
0.3	300	300	0.276	292	289	0.475	0.227	297	299	0.380
0.4	400	400	0.364	397	386	0.464	0.321	360	399	0.422
0.5	500	500	0.449	493	475	0.464	0.349	465	461	0.377
R^2 value			0.9994	0.9998	0.9998		0.9779	0.9942	0.9918	

[a] The calculation of the CM value is as follows: CM value = A × 1000/(B + C), A = the detected levels of citrinin (mg/kg); B = the detected levels of monacolin K lactone form (mg/kg); C = the detected levels of monacolin K acid form (mg/kg).

registered a depressed recovery rate of under 70% after quantitative analysis of CT. We hypothesized that CT compacted easily to combine with the starch molecules of the rice powder, and that the combinative ability is possibly stronger than the dissolubility of CT in ethanol. In addition, CT in RMR powder was found to have a higher recovery rate when ethanol or other solvents were used as compared with CT in unfermented rice powder. It is possible that the surface of RMR powder is covered by *Monascus* spp., which is likely to decrease the combination ability between starch and CT. Rice powder fermented by *Monascus* spp. may display an obvious difference in structure and character than unfermented rice.

Comparison with Synchronous Analysis Method and Published Individual Analysis Method

The synchronous analysis method provided a more rapid and convenient process than the traditional individual analysis method, but accuracy is more important than speed in an analytical method. For a comparison of accuracy between the 2 methods, the synchronous analysis method and traditional individual analysis method were evaluated by examining the concentrations of CT, MKL, and MKA and calculating R^2 and CM values from RMR with known concentrations. As shown in Table 2, a high linear relationship between true levels and detected levels of CT, MKL, and MKA was obtained when the R^2 value was close to 1.00. In addition, CT and MK levels expressed a ratio in the RMR. An accurate analysis method has to perform a ratio which is close to the true ratio of CT levels to MK levels from RMR. In this study, CM value, regarded as a ratio of CT levels to total MK levels in the RMR, was modulated to 0.5 by adding known concentrations of CT, MKL, and MKA to RMR powder. Therefore, the error of analysis method decreased when the CM value gained by detection of RMR became closer to 0.5. Table 2 indicates that the synchronous analysis method expressed higher R^2 square value (ranging from 0.9994 to 0.9998) and CM value (ranging from 0.452 to 0.475) than the traditional individual analysis method. Therefore, the synchronous analysis method had a higher accuracy than the traditional individual analysis method in this examination.

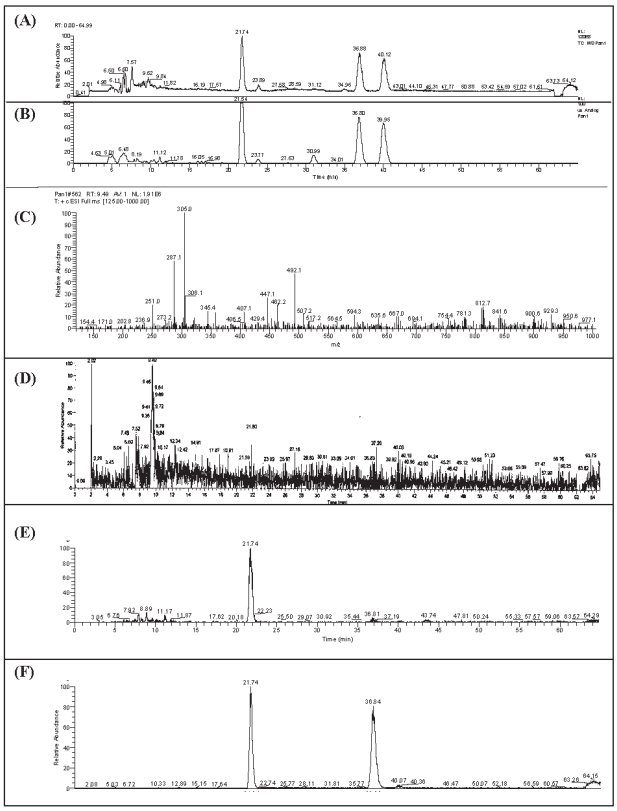

Figure 7. Chromatograms of red mold rice and mass spectra of selected peaks. (A) HPLC profile at 238 nm; (B) total ion current (TIC); (C) mass spectra of CT (M + 1 = 251.0) at 9.49 min; (D) selected-ion chromatogram (SIC) at *m/z* 250 (CT); (E) SIC at *m/z* 422.5–423.5 (acid form of monacolin K); (F) SIC at *m/z* 404.5–405.5 (lactone form of monacolin K).

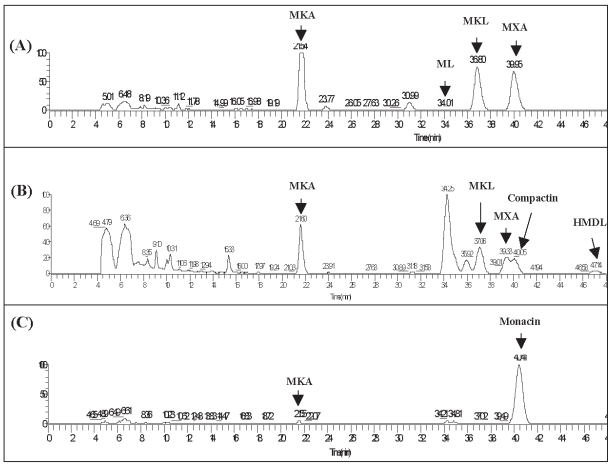

Figure 8. Chromatograms of red mold rice extract by LC/MS at 238 nm. (A) RMR1, (B) RMR2, (C) RMR3. CT = Citrinin; MKL = lactone form of monacolin K; MKA = acid form of monacolin K.

Identification by LC/MS

Ethanol extract of RMR powder is identified by LC/MS for the sake of the precision of the HPLC analysis. The chromatographic condition of LC/MS was modified from that of the HPLC. In addition to the flow rate being set at 0.5 mL/min, the chromatographic condition of LC/MS was identical to that of the HPLC established in this study. As shown in Figure 7, the chromatogram of LC/MS had a corresponding pattern compared to that of HPLC. CT, MKL, and MKA were detected in the mass spectra, which proves that the HPLC synchronous analysis method can be used to correctly detect CT, MKL, and MKA levels from ethanol extract of RMR powder.

As shown above, the synchronous HPLC analysis accurately obtained the retention time of CT, MKL, and MKA of RMR extract. LC/MS was used to further investigate and identify the corresponding compounds of the unknown peaks in the high-performance liquid chromatogram. In general, an identical condition of HPLC elution should lead to the same retention time in the same compound of various samples. Therefore, CT, MKL, and MKA will construct a similar base pattern on the chromatograms from various RMRs. LC/MS was used to identify the unknown peaks of the chromatograms obtained from 3 kinds of RMR with different metabolites. RMR1 fermented by a *Monascus* strain with high MK productivity included high MKA and MKL production. RMR2 was fermented by a *Monascus* selected from commercial RMR. RMR3 was observed to contain more yellow pigment in the exterior. Figure 8 shows that there are different patterns of UV chromatograms in various *Monascus* products. Monacolin L (ML), monacolin X acid form (MXA), 3α-hydroxy-3,5-dehydromonacolin L (HMDL), and compactin were detected by LC/MS in addition to MKA and MKL. The yellow pigment of RMR3 was proven to be monacin. We expect that the pattern can be used as a reference for deducing the corresponding compound of unknown peaks prior to further identification.

Table 3. Determination of CT, MKL, and MKA in commercial *Monascus* products

Sample	Country produced	Citrinin, μg/kg	Monacolin K lactone form, mg/kg	Monacolin K acid form, mg/kg
		Capsule		
C01	Taiwan	2067	28274	2976
C02	Taiwan	196	9846	590
C03	Taiwan	8921	11697	1163
C04	Taiwan	122	7589	2652
C05	United States	115	1033	128
C06	United States	11272	3249	115
C07	United States	449	3379	3886
		Red mold rice		
R01	Taiwan	12993	612	—[a]
R02	Taiwan	2679	214	38.0
R03	Taiwan	831	198	120
R04	Taiwan	1384	182	15.7
R05	China	618	15801	15577
R06	China	211	15010	3505
R07	Tailand	2271	1795	160
R08	Malaysia	10774	—	—
		Traditional food		
F01–edible oil	Taiwan	—	—	—
F02–sauce	Taiwan	14.26	2.55	—
F03–soybean sauce	Taiwan	9.36	—	—
F04–liquor	Taiwan	—	—	—
F05–vinegar	Taiwan	—	—	—
F06–vinegar	Japan	6.54	1.59	—

[a] — = Under detection limit.

Determination of CT, MKL, and MKA of Commercial Monascus Products

In this study, we used the synchronous analysis method to detect several commercial *Monascus* capsules and foods made in different countries. Table 3 shows that a high amount of total MK concentration ranging between 1161 and 31 250 mg/kg can be detected from these *Monascus* capsules, which is usually regarded as a functional food for lowering plasma and liver cholesterol levels. The *Monascus* processed foods are usually made by mixing a small amount of RMR with the food material, so that these processed foods contain lower MK and CT concentrations than the RMR. RMR and *Monascus* capsules made from dried *Monascus* fermented

rice contain different MK and CT concentrations by the character of *Monascus* species and fermented environment. These *Monascus*-related products can be purchased from markets in Taiwan and China and are chosen as the dietary supplement by more and more people because of the cholesterol-lowering agent MK. Importantly, several RMR and *Monascus* capsules such as C06, R01, and R08 sample include high CT concentration. This fact influences the health of people. The MK and CT concentrations of the *Monascus* capsule, RMR, and traditional food can be determined by the sampling examination.

In conclusion, the use of 100% ethanol (10 mL) at 60°C for 30 min can extract the largest amount of CT, MKL, and MKA from 1 g RMR powder. The extraction condition registered a recovery rate of 86.2, 94.0, and 91.8% in CT, MKL, and MKA, respectively. With regard to the optimal condition of HPLC, the peaks of CT, MKL, and MKA can be separated from noise by selecting acetonitrile–water–TFA (55 + 45 + 0.05) as the mobile phase of isocratic elution. In addition, the total length of time required for the analysis process, from extraction of the RMR sample to the data calculation, was less than 90 min. It is evident that the synchronous analysis method is a correct and quick method for detecting CT, MKL, and MKA levels at the same time. We expect that this analysis method will prove to be of great benefit in the determination of CT, MKL, and MKA in RMR.

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