FOOD CHEMICAL CONTAMINANTS

Synchronous High-Performance Liquid Chromatography with a Photodiode Array Detector and Mass Spectrometry for the Determination of Citrinin, Monascin, Ankaflavin, and the Lactone and Acid Forms of Monacolin K in Red Mold Rice

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The *Monascus* fermentation product red mold rice (RMR) has been found to contain the cholesterol-lowering agent monacolin K (MK) in both its lactone (MKL) and acid (MKA) forms and the mycotoxin citrinin (CT). The yellow pigments in RMR, namely, monascin (MS) and ankaflavin (AK), have been reported to exhibit antimetastatic and antiangiogenic activities. Currently, MK and these yellow pigments are usually detected in RMR by different analytical methods that are inconvenient, expensive, and time-consuming. The goal of this study was to establish a rapid, synchronous analytical method for determination of the MKA, MKL, MS, AK, and CT levels in RMR. MKA, MKL, MS, AK, and CT were extracted by the same extraction method, then separated by RP-HPLC with a C_{18} column. The effluent from the column was passed through a photodiode array detector and then introduced directly into a fluorescence detector. The results showed that high recovery rates of MKA, MKL, MS, AK, and CT are possible if RMR powder is extracted with 75% ethanol (10 mL) at 80°C for 30 min. With regard to the optimal conditions of the HPLC, the peaks of MKA, MKL, MS, AK, and CT can be clearly separated from any noise peaks by isocratic elution with a mobile phase comprising 0.05% trifluoroacetic acid in acetonitrile–water (62.5 + 37.5, v/v).

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Products obtained by fermentation with molds of the genus *Monascus* have been found to contain several bioactive compounds. They have been used as a traditional food in East Asia for several centuries. Among the useful secondary metabolites in *Monascus* fermentation, monacolin K (MK) has been proven to be an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the cholesterol biosynthesis pathway (1, 2). *Monascus*-fermented rice, known as red mold rice (RMR), has gradually become a popular functional food for antihyperlipidemia (3, 4) and antihypertension (5, 6). In a previous study, we found that red mold dioscorea contained more monascin (MS), a yellow pigment with anti-inflammatory potential. MS is significantly formed and substituted for the red pigment (monascorubramine) as the major pigment of red mold dioscorea (6, 7). Inflammation may cause hyperlipidemia, atherosclerosis, and hypertension, and the anti-inflammatory ability of MS has been proven to prevent hypertension (6).

Although *Monascus*-fermented products have been used as dietary supplements in East Asia for thousands of years, their use is still restricted, and they are not accepted in many countries. This is because citrinin (CT) has previously been identified as a secondary metabolite produced by various fungi, including *Monascus* spp. (8), and it is a known hepato-nephrotoxin to several species and has been implicated as the potential causative agent of endemic Balkan nephropathy in humans (9, 10). The action of CT at the cellular level is characterized by its accumulation in the mitochondria and interference with the electron transport system (11).

Recently, the yellow pigments of *Monascus*, namely, MS and ankaflavin (AK), have been reported to have

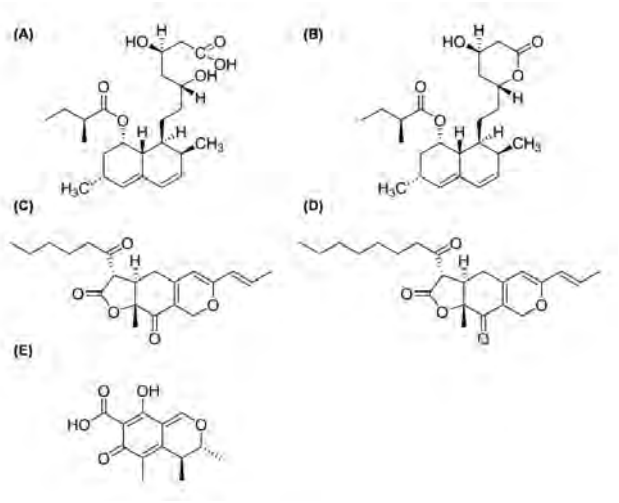

Figure 1. Structures of (A) the acid form of monacolin K (MKA), (B) the lactone form of monacolin K (MKL), (C) monascin (MS), (D) ankaflavin (AK), and (E) citrinin (CT).

cytotoxic/cytostatic activities (12, 13), although they were not found to be significantly cytotoxic toward rat hepatocytes in vitro (14). Our previous study showed that monacolin K (also known as mevinolin; MK), MS, and AK inhibited the metastatic ability of murine Lewis lung carcinoma cells (LLC) by reducing the levels of serum vascular endothelial growth factor (15). RMR extract could contain several other compounds with antimetastatic and antiangiogenic effects (15). Therefore, RMR extract may serve as a nontoxic, natural chemopreventive or antineoplastic agent in adjuvant cancer chemotherapy (15).

In the present study, MK and the yellow pigments were identified using different analytical methods (16, 17). Commercial *Monascus* products have attracted substantial attention because of their levels of MS, AK, CT, and the lactone and acid forms of MK (MKL and MKA, respectively). Figure 1 shows the structures of MKA, MKL, MS, AK, and CT from RMR. We previously developed a synchronous HPLC analytical method for the quantification of MK and CT. This method combines HPLC-UV detection for MK and HPLC-fluorescence detection for CT (16). The purpose of this study was not only to establish a universal, sensitive, and rapid synchronous analytical method but also to identify the optimal extraction conditions for determining the concentrations of MKA, MKL, MS, AK, and CT from HPLC chromatograms. MKA, MKL, MS, and AK are detected at the UV wavelength of 234 nm. Therefore, this study describes a synchronous analytical system for determining the levels of MKA, MKL, MS, AK, and CT in RMR by using the same C_{18} RP chromatographic column and connecting a fluorescence detector with a UV detector.

Experimental

Standards, Chemicals, and Test Materials

(**a**) *Standards*.—MK and CT were purchased from Sigma Chemical Co. (St. Louis, MO). In general, MK has two forms: MKL and MKA. The commercial standard of MK is MKL, which can be transformed to MKA by alkalization. Therefore, standard MKA solution was prepared as follows: MKL was dissolved in 0.1 M sodium hydroxide ethanolic solution in a concentration range of 100–1000 mg/L. Standard solutions of CT and MKL were prepared by dissolving CT and MKL in ethanol in a concentration range of 100–500 μg/kg and 100–1000 mg/kg, respectively. MS and AK were obtained as described previously with some modifications (18). They were extracted by acetone and then fractionated by conventional packed-column chromatography. After purifying by pre-HPLC, pure compounds MS and AK were produced. The purity of MS and AK was verified by NMR spectrometry and electrospray ionization (ESI)-mass spectrometry analyses.

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

(**c**) *Monascus-related food products*.—RMR and *Monascus* capsules were purchased from the local market in Taipei, Taiwan.

HPLC Synchronous Analytical System

The synchronous analytical system for CT, MKA, MKL, MS, and AK comprised an injector, mobile phase pump, C_{18} column, photodiode array (PDA) detector, and fluorescence detector. In order to detect these five components in the same analysis, the fluorescence detector was connected to the system via the PDA detector. Thus, the effluent from the C_{18} column was passed through the PDA detector and then directly introduced into the fluorescence detector. CT was detected by the fluorescence detector with excitation and emission wavelengths set at 330 and 500 nm, respectively (19). MKA, MKL, MS, and AK were detected by a PDA detector with the wavelength set at 234 nm (17, 20).

(**a**) *Column*.—LUNA C_{18}, 25 cm × 4.6 mm id, 5 μm particle size (Phenomenex, Torrance, CA).

(**b**) *Intelligent sampler*.—AS-2055 plus (Jasco, Tokyo, Japan).

(**c**) *Quaternary gradient pump*.—PU-2089 plus (Jasco).

(**d**) *Multiwavelength detector*.—MD-2010 plus (Jasco).

(**e**) *Intelligent fluorescence detector*.—FP-2020 plus (Jasco).

NMR System

Gemini, 200 MHz, FT-NMR spectrometer (Varian Inc., Palo Alto, CA).

HPLC/Mass Spectrometry System

(**a**) *HPLC*.—Finnigan MAT Spectra LC system (Thermo Scientific, San Jose, CA), including a quaternary pump, online degasser, column heater compartment, and autosampler.

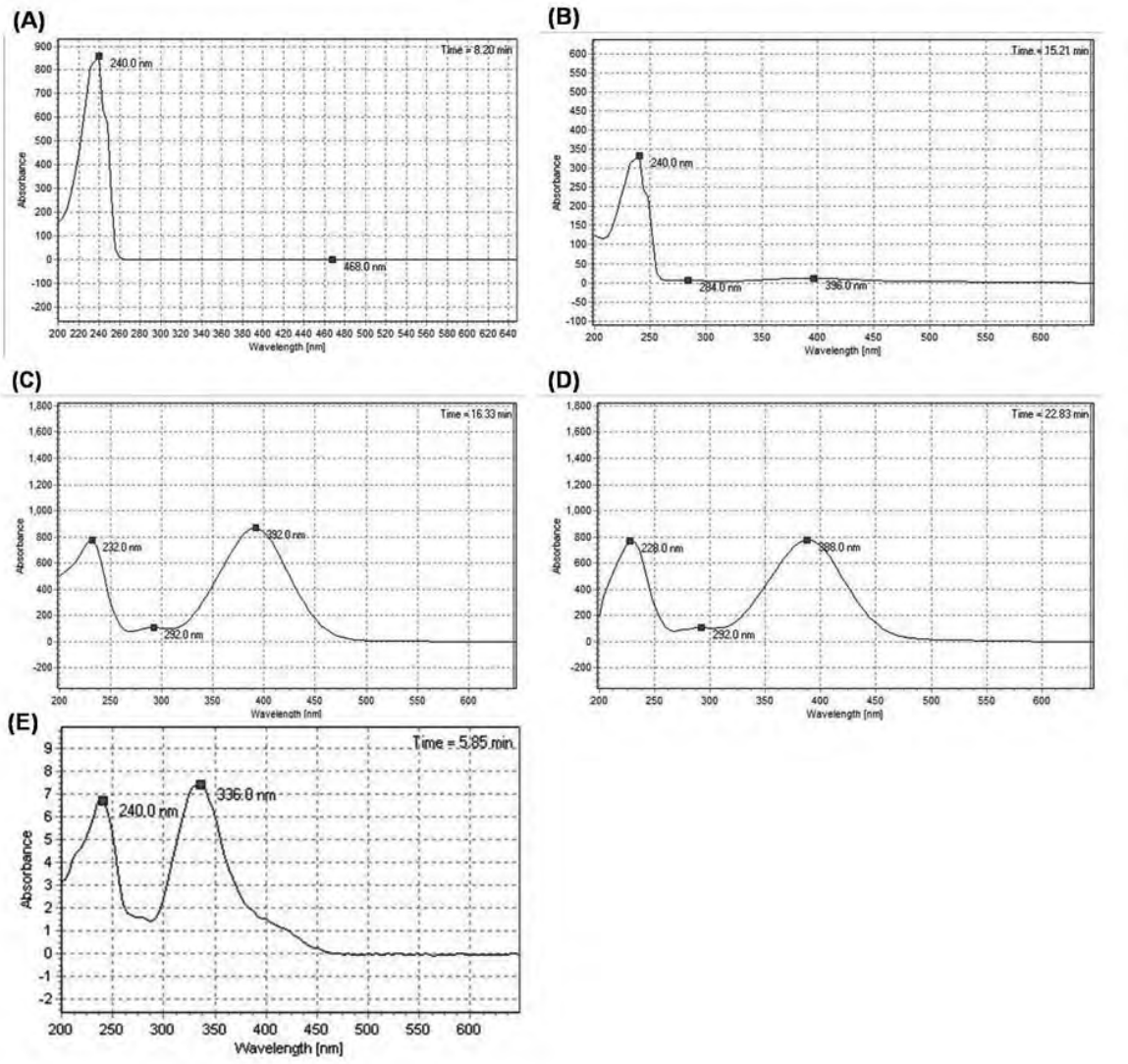

Figure 2. UV spectra of (A) MKA, (B) MKL, (C) MS, (D) AK, and (E) CT.

(**b**) *Mass spectrometry*.—Finnigan MAT LCQ mass spectrometer (Thermo Scientific) consisting of an ESI interface and an ion-trap mass analyzer.

(**c**) *HPLC/mass spectrometry software*.—The software controlling the equipment and for data treatment was Xcalibur Core Data System Software Revision 1.0 (Xcalibur Software Inc., Herndon, VA).

HPLC Conditions in the Synchronous Analytical System

CT, MKA, MKL, MS, and AK were analyzed by HPLC on the Phenomenex Luna C_{18} column by isocratic elution using 0.05% TFA in acetonitrile–water (62.5 + 37.5, v/v) as the mobile phase. The flow rate was set at 1.0 mL/min. TFA was selected as the mobile phase acidifier based on our previous study (7).

HPLC/Mass Spectrometry Conditions

The HPLC/mass spectrometry conditions were modified from the conditions used for HPLC. The separation by HPLC/mass spectrometry was carried out using an RP C_{18} column with isocratic elution at a flow rate of 0.5 mL/min. The composition of the mobile phase was the same as that of the optimal mobile phase in the HPLC analysis. All 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 A.U.; and auxiliary gas (N_2) flow, 10 A.U. The ESI interface and mass spectrometry parameters were optimized to obtain maximum sensitivity unit resolution.

Extraction Experiment

The basic process of extraction was as follows: RMR powder (1 g) was extracted with 10 mL extraction solvent at 60°C for 30 min with shaking (7). Various solvents (methanol, 95% ethanol, 75% ethanol, 50% ethanol, acetonitrile, ethyl acetate, acetone, and hexane) at various extraction temperatures (30, 40, 50, 60, 70, and 80°C) and for various times (5, 15, 30, 45, and 60 min) were used, with

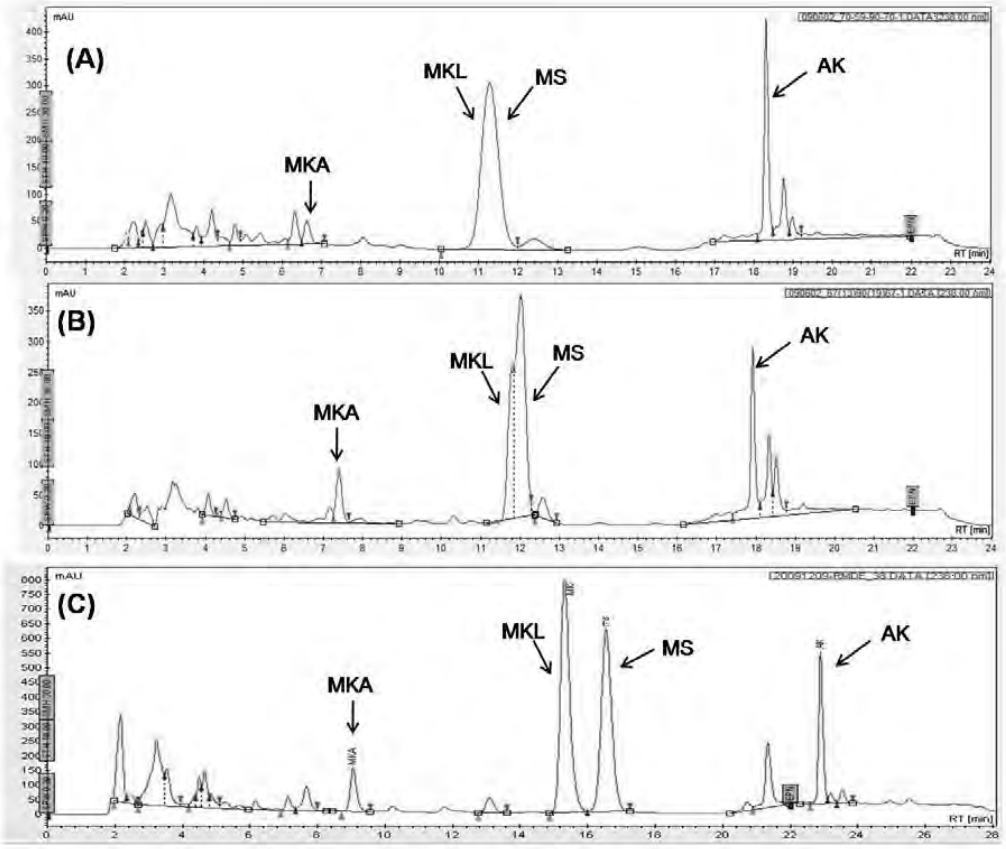

Figure 3. Effect of various mobile phase compositions, including (A) 0.05% TFA in acetonitrile–water (67.5 + 32.5, v/v), (B) 0.05% TFA in acetonitrile–water (65 + 35, v/v), and (C) 0.05% TFA in acetonitrile–water (62.5 + 37.5, v/v), on peak separation in the chromatograms of MKA, MKL, MS, and AK obtained using the synchronous HPLC analytical method.

shaking, to determine the optimal extraction conditions. After extraction, a 1 mL suspension was transferred to a new Eppendorf tube and evaporated to dryness in a glass desiccator under vacuum. Then, 1 mL acetonitrile was added to the tube, followed by filtration with a 0.45 μm pore size filter and HPLC analysis.

Recovery Experiment

RMR extract analyzed by the method in this study with known amounts of MKA (124.9 mg/L), MKL (213.3 mg/L), MS (1210.8 mg/L), AK (190.5 mg/L), and CT (270 μg/L) was obtained by multiple analyses using the current method and was selected as the control sample for the recovery experiment. MKA (1.875, 3.75, 7.5, 15, or 30 mg/L), MKL (2.5, 5, 10, 20, or 40 mg/L), MS (12.5, 25, 50, 100, or 200 mg/L), AK (2.5, 5, 10, 20, or 40 mg/L), and CT (7.5, 15, 30, 60, or 120 μg/L) at various concentrations were added to the same test RMR sample powder (1 g) and then evaporated to dryness under vacuum. The test RMR powder sample with extra MKA, MKL, MS, AK, and CT was examined under optimal conditions using the synchronous analytical method. The recovery rate was calculated as follows:

$$\text{Recovery rate } (\%) = \left[\left(A' - B\right)/(A)\right] \times 100\%$$

where A′ = total level of the target compound in the test RMR sample determined by the synchronous analytical method; A = level of the added target compound; and B = original level of the target compound in the control RMR sample detected by the synchronous analytical method (MKA 1249 mg/kg, MKL 2133 mg/kg, MS 12 108 mg/kg, AK 1905 mg/kg, and CT 2700 μg/kg).

Statistical Analysis

Data were expressed as the mean ± SD. The statistical significance of the extraction experiment of the test compounds was determined by one-way analysis of variance (ANOVA) using the general linear model procedure of the SPSS software (SPSS Institute Inc., Chicago, IL), followed by ANOVA with Duncan's test. Differences with $P < 0.05$ were considered statistically significant.

Results and Discussion

Identification by HPLC-PDA

The ethanol RMR extract was examined by HPLC/mass spectrometry to test the results of the HPLC analysis. As shown in Figure 2, the UV spectra of MKA, MKL, MS, AK, and CT were measured using HPLC-PDA. The spectrum of

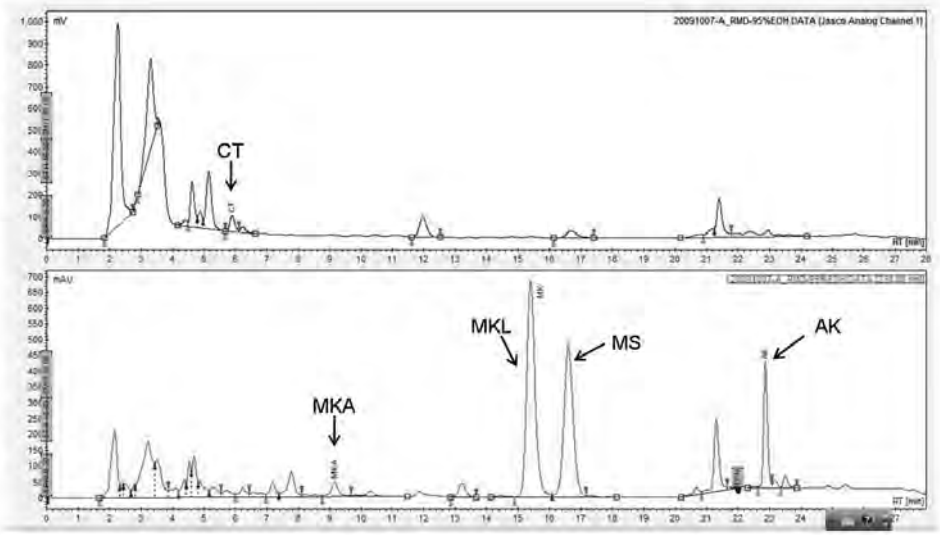

Figure 4. Chromatograms of MKA, MKL, MS, AK, and CT obtained under optimal conditions with the synchronous HPLC analytical method involving both UV (bottom) and fluorescence (top) detectors.

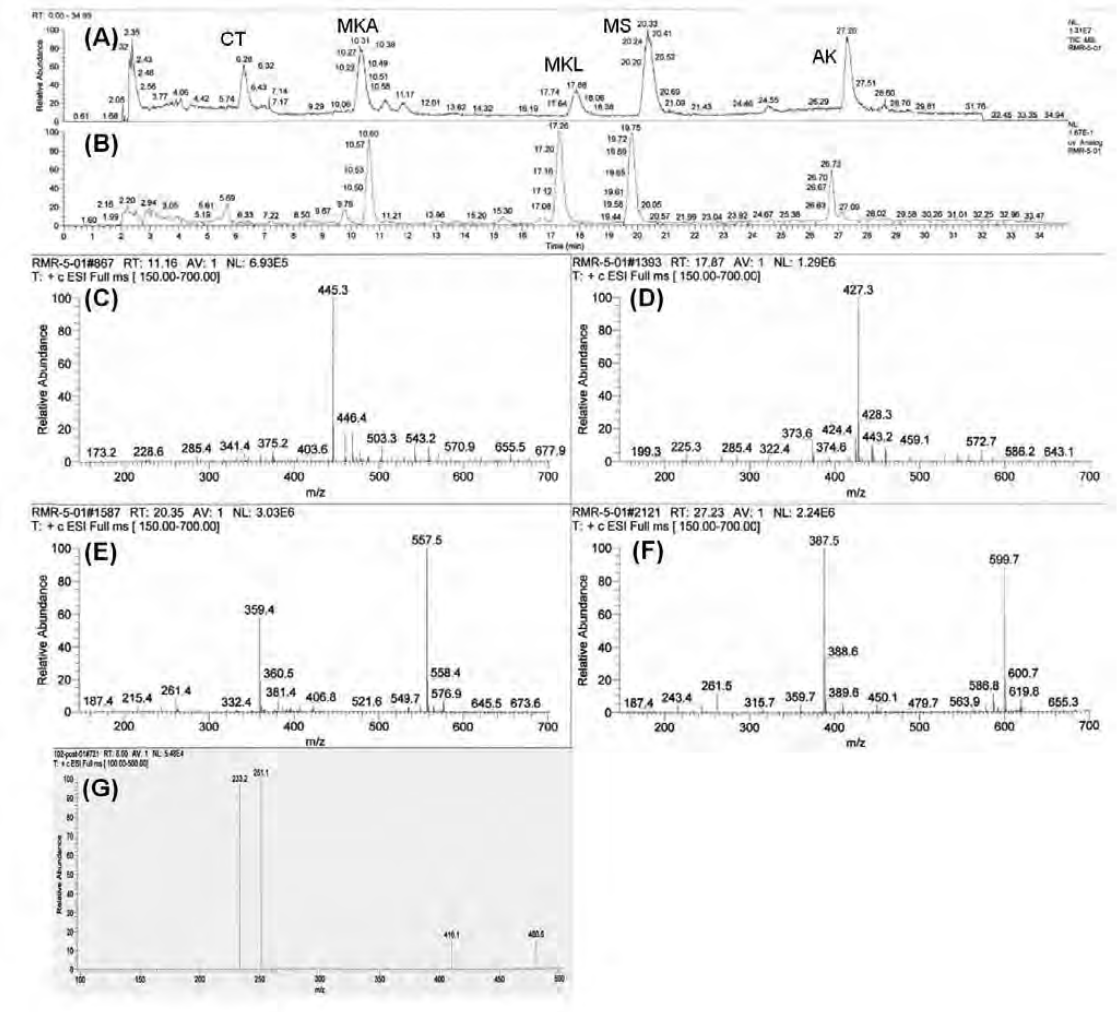

Figure 5. Chromatograms of RMR and mass spectra of selected peaks: (A) total ion current (TIC); (B) HPLC profile at 234 nm; (C) MKA, (D) MKL, (E) MS, (F) AK, and (G) CT.

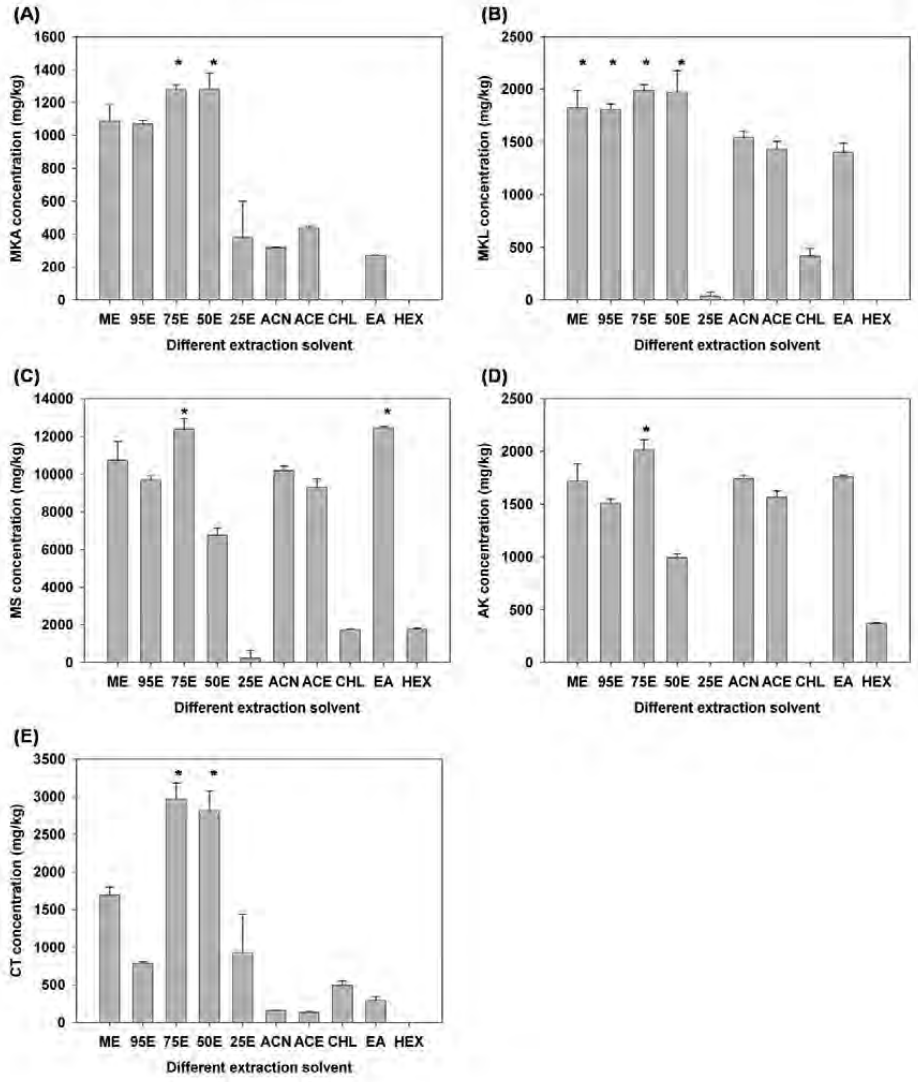

Figure 6. Effect of various extraction solvents, including methanol (ME), 95% ethanol (95E), 75% ethanol (75E), 50% ethanol (50E), 25% ethanol (25E), and acetonitile (ACN), on the extraction levels of (A) MKA, (B) MKL, (C) MS, (D) AK, and (E) CT under the basic conditions (60°C, 100 rpm, 30 min). Each data point represents a sample measured in triplicate, and values are reported as the mean ± SE. * = More significant differences than other groups ($P < 0.05$). There is no significant difference among the bars with an asterisk ($P < 0.05$).

MS has two maximum UV absorption wavelengths: 232 and 392 nm. AK also has two maximum UV absorption wavelengths of 228 and 388 nm. Both MKA and MKL have one maximum UV absorption wavelength at 240 nm. However, they have similar maximum wavelengths near 240 nm. Therefore, using 240 nm as the detection wavelength in the synchronous analytical method, we determined MKA, MKL, MS, and AK in a single analysis.

Effect of Mobile Phase Composition on HPLC

The ratio of acetonitile to distilled water influenced the separation of selected peaks from noise peaks in HPLC. In order to investigate the optimal conditions for separating the peaks of MKA, MKL, MS, and AK, aqueous acetonitile at 62.5, 65.0, and 67.5% was used as the mobile phase. Because MKA, MKA, MS, and AK have a similar maximum UV absorption wavelengths (about 234 nm), they must be well separated to avoid the problem of overlapping peaks under different conditions. As shown in Figure 3, good separation for the four compounds was obtained when 62.5% aqueous acetonitile was used as the mobile phase. Figure 4 shows the optimal profile obtained using the synchronous analytical method (top: fluorescence detector pattern; bottom: PDA detector pattern at 234 nm).

Identification of Compounds by HPLC/Mass Spectrometry

As stated above, the ethanol RMR extract was analyzed by HPLC/mass spectrometry to verify the proper peak assignments in the HPLC analyses. HPLC/mass spectrometry

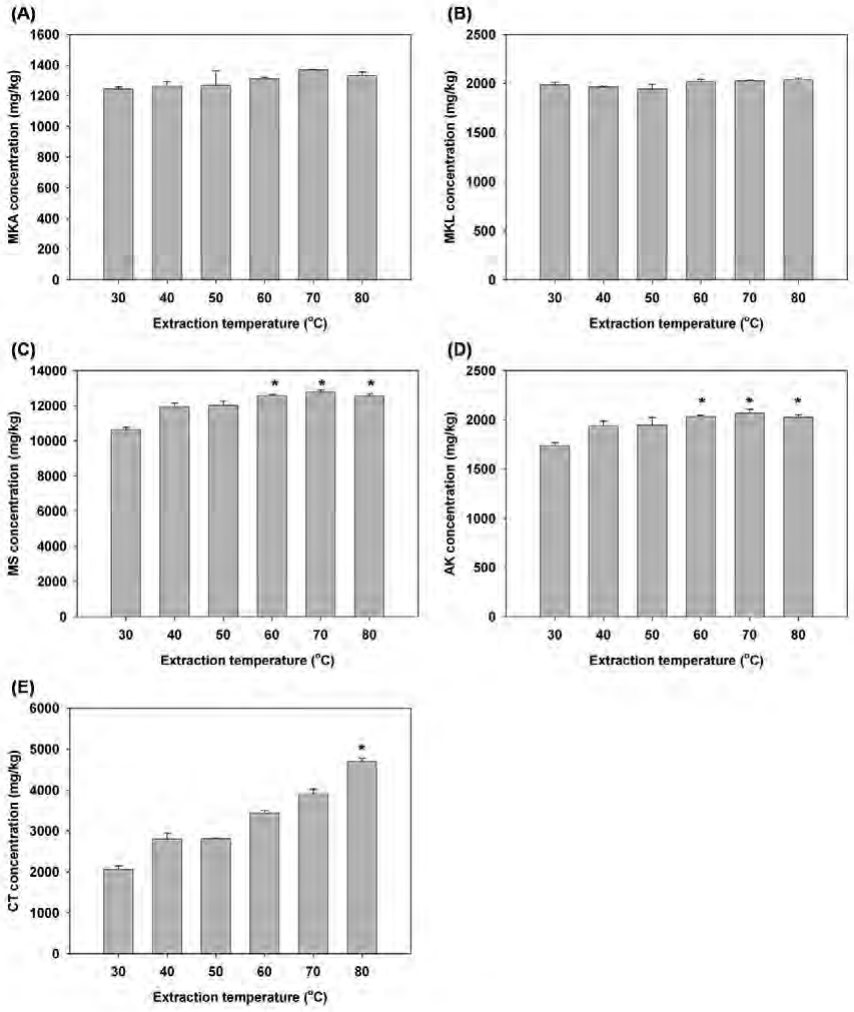

Figure 7. Effect of various extraction temperatures on the extraction levels of (A) MKA, (B) MKL, (C) MS, (D) AK, and (E) CT under the basic conditions (100 rpm, 30 min). Each data point represents a sample measured in triplicate, and values are reported as the mean ± SE. * = More significant differences than other groups ($P < 0.05$). There is no significant difference among the bars with an asterisk ($P < 0.05$).

conditions were identical to those for HPLC, except that the flow rate was set to 0.5 mL/min. As shown in Figure 5, the HPLC/mass spectrometry chromatogram had a pattern corresponding to that of the HPLC chromatogram. MKA, MKL, MS, AK, and CT were detected by the mass spectra, which proves that the HPLC-based synchronous analytical method can be used reliably to determine the MKA, MKL, MS, AK, and CT levels in ethanolic RMR extract.

Extraction Solvent

Investigation of the effect of extraction 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 acetonitile. Various organic solvents in which MKA, MKL, MS, AK, and CT dissolve were used to determine the effect on RMR extraction. The results (Figure 6) show that MKA was extracted in the highest amount when 75 and 50% ethanol were used as the extraction solvent. The use of methanol and 95, 75, or 50% ethanol as the extracting solvent yielded the maximum amount of MKL. High levels of MS were extracted when 75% ethanol and 100% ethyl acetate were used as the extraction solvent. Further, the AK was extracted at the highest level when 75% ethanol was used as the extracting solvent. Lastly, 75 and 50% ethanol yielded the best extraction of CT. Thus, the extraction levels of CT, MKA, MKL, MS, and AK varied significantly among the different solvents used, especially 25% ethanol, acetone, acetonitile, chloroform, and hexane, which were poor extraction solvents compared to the rest. Among the solvents, 75% ethanol extracted the highest amounts of all of the test compounds from RMR; therefore, it was selected as the ideal solvent for extracting these five compounds.

Extraction Temperature

Heat is usually used to accelerate the breakage of cells and obtain higher levels of extraction, especially for intracellular samples. Therefore, the RMR was placed in 75% ethanol and heated for 30 min at 30, 50, 60, 70, and 80°C. As shown in

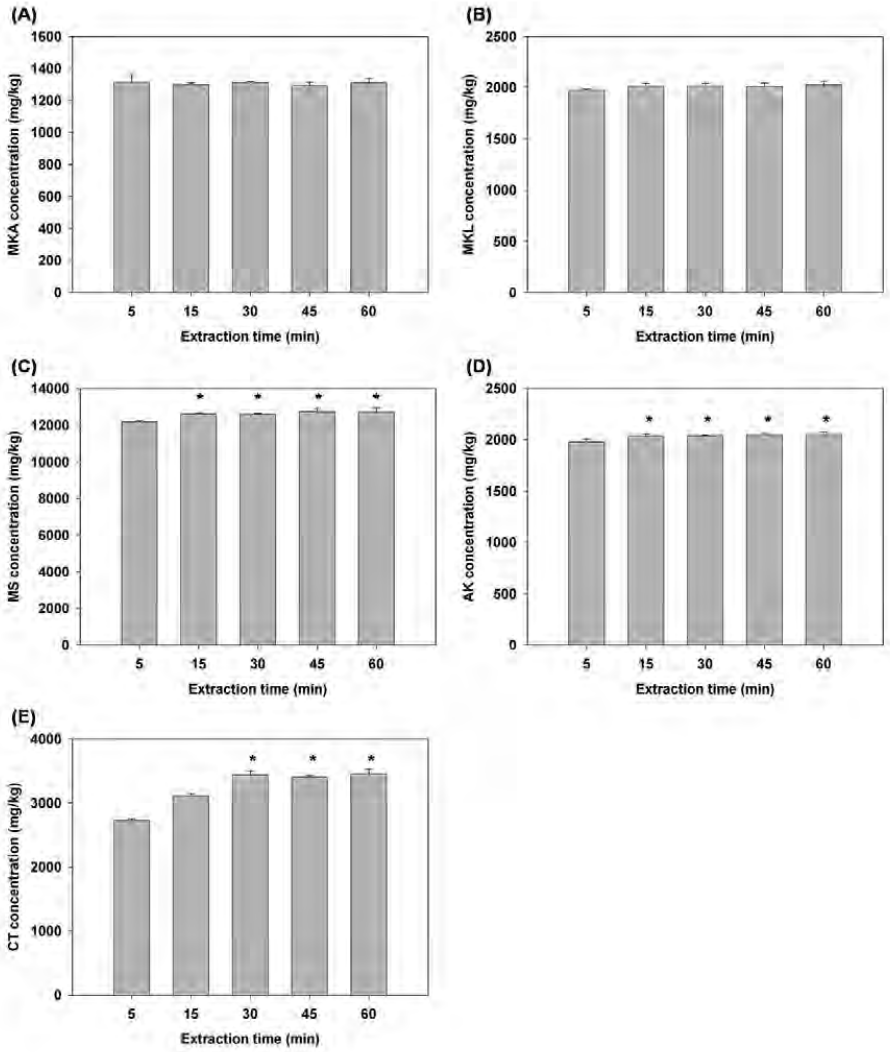

Figure 8. Effect of various extraction times on the extraction levels of (A) MKA, (B) MKL, (C) MS, (D) AK, and (E) CT under the basic conditions (80°C, 100 rpm). Each data point represents a sample measured in triplicate, and values are reported as the mean ± SE. * = More significant differences than other groups ($P < 0.05$). There is no significant difference among the bars with an asterisk ($P < 0.05$).

Figure 7, heating increased the extracted levels of MS, AK, and CT. MS and AK were best extracted at 60, 70, and 80°C, and CT at 80°C. The extraction temperature was set at 80°C because the extraction levels of all five compounds were best at this temperature. A comparison between heat and sonic extractions for MKA, MKL, MS, AK, and CT are shown in Figure 7. The extraction time was set at 30 min because the extraction levels of MKA, MKL, MS, AK, and CT were previously reported to be the best at this time (16).

Extraction Time

Ideally, an extraction procedure should yield the largest amount of the target compounds in the shortest time, thereby reducing both the time required and cost. Figure 8 shows the extraction levels of the five compounds at different extraction times. The extracted levels of MKA and MKL did not vary significantly at different extraction times. However, those of MS and AK did vary significantly with the extraction time, especially 15, 30, 45, and 60 min. The extraction level of CT also varied significantly with extraction time, especially 30, 45, and 60 min. The maximum amounts of MKA, MKL, MS, AK, and CT were extracted from the RMR at 30 min; increasing the extraction time did not increase the extraction levels of the target compounds.

Extraction Dilution Factor

The RMR was immersed in 75% ethanol, heated at 80°C, and extracted at dilution factors of 5, 10, and 20 for 30 min. As shown in Figure 9, the dilution factor affected the extraction levels of MKL, AK, and CT. A dilution factor of 5 or 10 yielded better extraction levels of MKL and AK, and a dilution factor of 10 yielded the best extraction level for CT. In order to establish a simple and convenient analytical

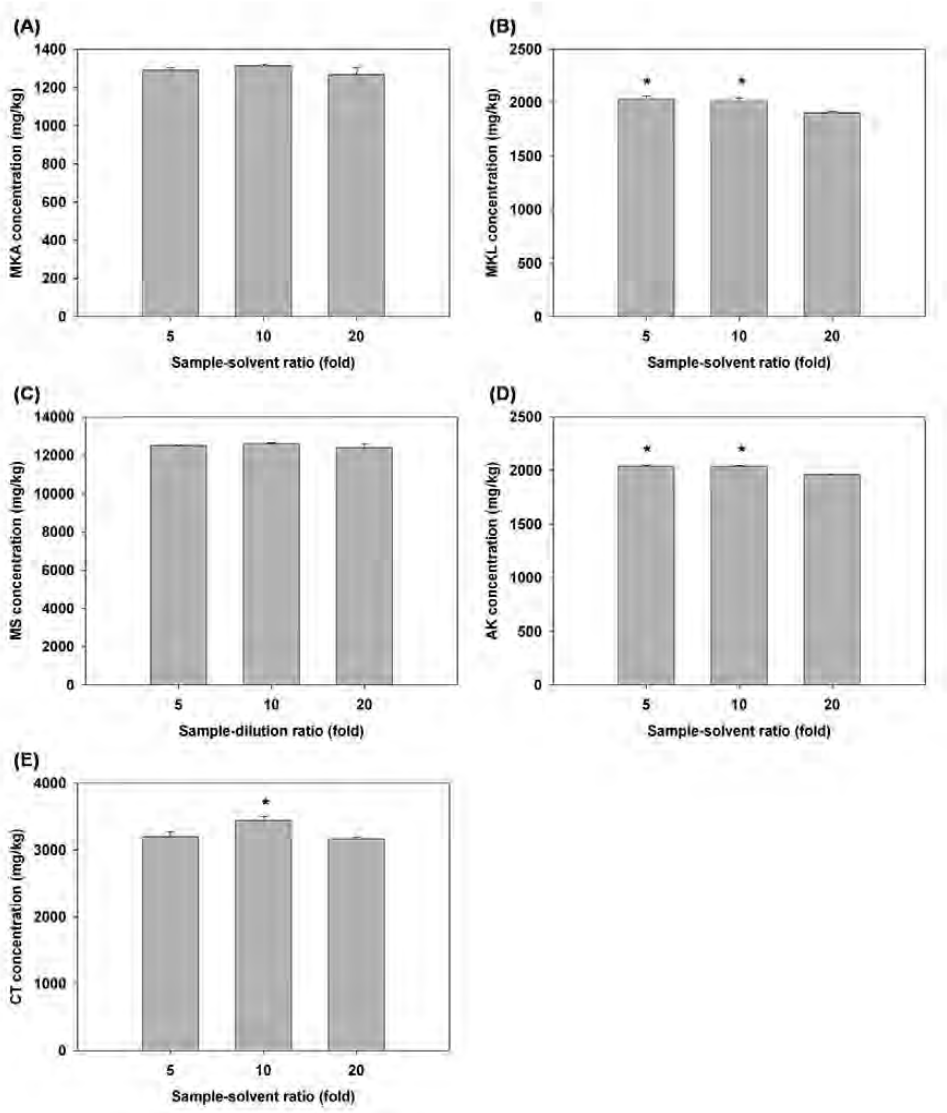

Figure 9. Effect of various extraction sample-solvent ratios on the extraction levels of (A) MKA, (B) MKL, (C) MS, (D) AK, and (E) CT under the basic conditions (80°C, 100 rpm, 30 min). Each data point represents a sample measured in triplicate, and values are reported as the mean ± SE. * = More significant differences than other groups ($P < 0.05$). There is no significant difference among the bars with an asterisk ($P < 0.05$).

method, the RMR was immersed in 75% ethanol, heated at 80°C, and extracted at a dilution factor of 10 for 30 min.

Standard Curve and Recovery Experiment

According to the results of the chromatographic and extraction condition studies, a mobile phase containing 0.05% TFA in acetonitrile–water (62.5 + 37.5, v/v) was selected for the HPLC analysis. The standard curves of CT, MKA, MKL, MS, and AK were then investigated. The R^2 values calculated from the standard curves of MKA, MKL, MS, AK, and CT were 0.9996, 0.9969, 0.9999, 0.9998, and 0.9998, respectively (data not shown).

In order to investigate the recovery rate of the extraction process, standard solutions of MKA, MKL, MS, AK, and CT or distilled water were added to RMR powder that had known concentrations of MKA, MKL, MS, AK, and CT based on prior analysis to be used as the test sample. The test samples were extracted and examined by the quantitative analytical method established in this study. The control RMR sample was used as the starting material without extra MKA, MKL, MS, AK, and CT. The concentrations of the added MKA, MKL, MS, AK, and CT in the test RMR sample were obtained by subtracting the original concentration from the total examined concentration. Table 1 shows that the average recovery rate obtained with the extraction method established in this study was 92.99, 95.61, 95.91, 92.63, and 91.12% for MKA, MKL, MS, AK, and CT, respectively.

Quantification of MKA, MKL, MS, AK, and CT in Commercial Monascus Products

As mentioned above, our synchronous HPLC analytical method accurately determined the retention times of MKA,

Table 1. Recovery rate of CT, MKA, MKL, MS, and AK from RMR

Amount added					Recovery rate, %				
Citrinin, μg/L	Monacolin K acid form, μL	Monacolin K lactone form, mg/L	Monascin, mg/L	Ankaflavin, mg/L	Citrinin	Monacolin K, acid form	Monacolin K, lactone form	Monascin	Ankaflavin
120	30	40	200	40	88.40 ± 0.65	91.20 ± 0.08	95.27 ± 2.18	97.22 ± 1.12	93.61 ± 0.75
60	15	20	100	20	90.48 ± 3.41	95.59 ± 0.03	94.42 ± 1.86	94.63 ± 1.23	92.36 ± 1.25
30	7.5	10	50	10	95.03 ± 3.09	94.16 ± 0.01	95.41 ± 1.29	94.69 ± 0.76	94.01 ± 0.10
15	3.75	5	25	5	96.66 ± 2.85	91.54 ± 0.02	96.58 ± 1.08	96.86 ± 0.49	92.04 ± 3.16
7.5	1.875	2.5	12.5	2.5	85.02 ± 5.72	92.44 ± 0.01	96.37 ± 1.40	96.15 ± 1.50	91.13 ± 1.95
Average					91.12 ± 4.78	92.99 ± 0.02	95.61 ± 0.88	95.91 ± 0.40	92.63 ± 1.17

MKL, MS, AK, and CT in the RMR extracts. HPLC/mass spectrometry was used to identify the compounds corresponding to the unknown peaks in the HPLC chromatograms. In general, identical conditions of elution in HPLC should yield the same retention time for a single compound in different samples. Therefore, MKA, MKL, MS, AK, and CT from various RMRs should produce a similar base pattern on the chromatograms, provided the HPLC conditions used for the analysis are kept constant. HPLC/mass spectrometry was used to identify the unknown peaks in the chromatograms obtained from five kinds of RMR with different metabolites. RMR1 was purchased from a normal market. RMR2 was fermented by a *Monascus* sp. selected from a commercially available RMR that had high amounts of

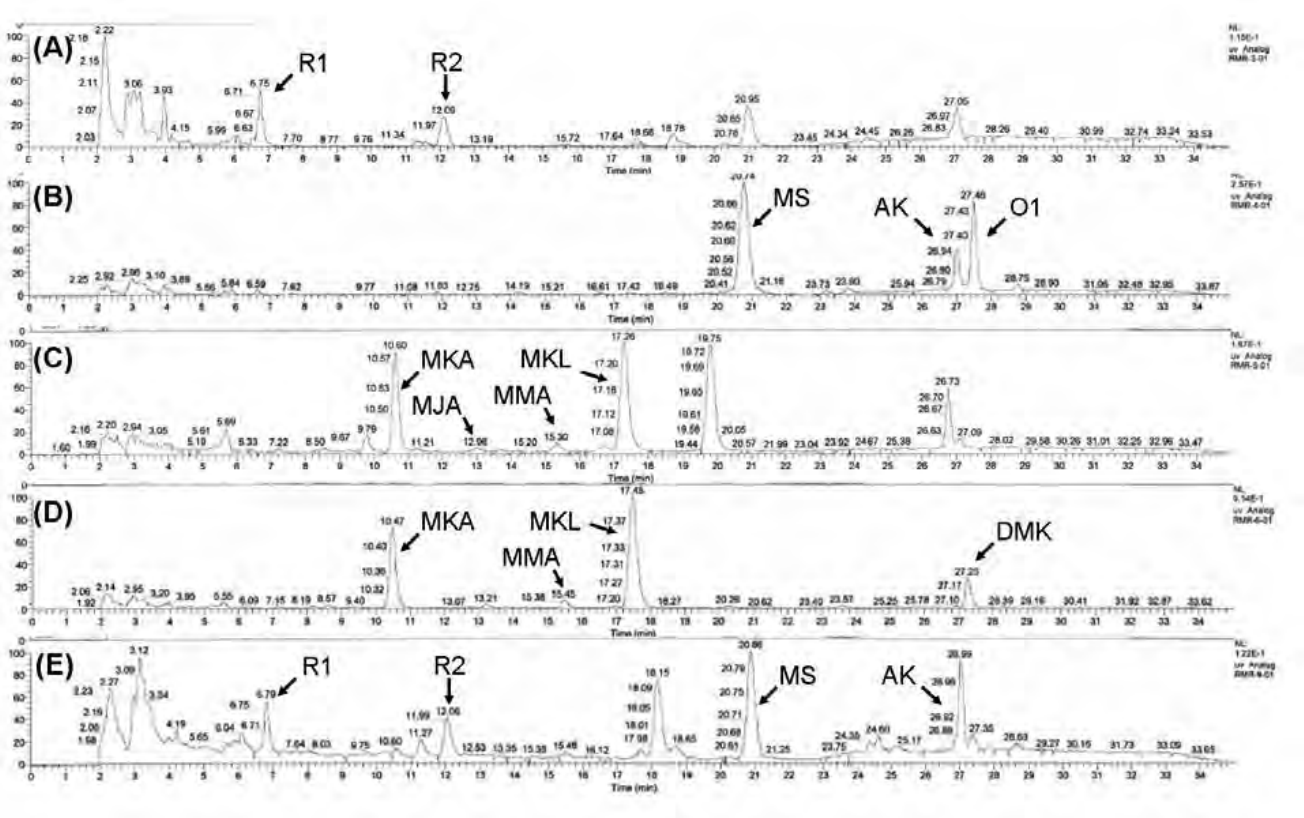

Figure 10. Chromatograms of commercial RMR extract obtained by HPLC using a UV detector with the wavelength set at 234 nm. (A) RMR1, (B) RMR2, (C) RMR3, (D) RMR4, and (E) RMR5. CT = Citrinin, MKA = the acid form of monacolin K, MKL = the lactone form of monacolin K, MS = monascin, AK = ankaflavin, R1 = rubropunctamine (red pigment), R2 = monascorubramine (red pigment), O1 = monascorubrin (orange pigment), MJA = the acid form of monacolin J, MMA = the acid form of monacolin M, and DMK = dehydromonacolin K.

Table 2. Quantification of CT, MKL, and MKA in commercial *Monascus* products

Sample	Country of production	Citrinin, μg/kg	Monacolin K, acid form, mg/kg	Monacolin K, lactone form, mg/kg	Monascin, mg/kg	Ankaflavin, mg/kg
			Capsule			
C01	Taiwan	1788	2576	6417	—[a]	—
C02	Taiwan	14099	—	4572	3790	615
C03	Taiwan	439	644	8860	—	—
C04	Taiwan	—	2333	2033	—	73
C05	Taiwan	—	520	8580	—	—
C06	Taiwan	—	1270	1870	11980	1890
C07	China	241	411	1289		—
C08	China	1260	1960	2360		—
C09	China	0	15838	15405		—
C10	Japan	98	2430	12678		—
			Red mold rice			
R01	Taiwan	5742	—	—	2573	1653
R02	Taiwan	27000	—	—	2770	725
R03	China	13550	—	—	5040	1465
R04	China	—	—	—	—	—
R05	China	49	—	—	9332	2383

[a] — = Under detection limit.

orange pigment. RMR3 was a commercial functional RMR capsule containing MKA, MKL, MS, and AK. RMR4 was produced by fermentation with a *Monascus* strain with high MK productivity, including MKA and MKL. Finally, RMR5 was observed to contain more yellow pigment. Figure 10 shows the different patterns of the UV chromatograms produced by the different *Monascu*s products. In addition to MKA, MKA, MS, and AK, the acid form of monacolin J (MJA), the acid form of monacolin M (MMA), rubropunctamine (R1), monascorubramine (R2), monascorubrin (O1), and dehydromonacolin K (DMK) were detected by HPLC/mass spectrometry. We expect that the patterns can be used as references for identifying the compounds corresponding to unknown peaks prior to further identification.

We used the synchronous analytical method to examine the compounds in several commercial *Monascus* capsules and food products made in different countries. Table 2 shows that these *Monascus* capsules, which are usually regarded as functional foods for lowering plasma and liver cholesterol, contained high concentrations of MK, ranging between 1700 and 31 243 mg/kg, and high concentrations of the yellow pigments. RMR and *Monascus* capsules made from dried *Monascus*-fermented rice contained different concentrations of MK, MS, AK, and CT depending on the *Monascus* species and fermentation conditions. These *Monascus*-fermented products can be purchased from markets in Taiwan and China and are being selected as dietary supplements by an increasing number of people because of the cholesterol-lowering activity of MK. Importantly, several RMR and *Monascus* capsules, such as the C02, R02, and R03 samples, contained high levels of CT, which affect human health. The MK, MS, AK, and CT concentrations in *Monascus* capsules and RMR can be determined by the synchronous quantitative analytical method described in this study.

Previous studies have shown the individual qualitative and quantitative analysis of MK (acid and lactone forms) and the yellow pigments MS and AK (16, 17). However, no analytical method is available to qualitatively and quantitatively determine MKA, MKL, MS, AK, and CT simultaneously. In this study, we established such a synchronous analytical method for MKA, MKL, MS, AK, and CT to identify the peaks of MK and MS, which have nearly identical retention times and maximum UV absorption wavelengths (230–240 nm) in HPLC and to establish the optimal extraction conditions for these five compounds from commercial RMR samples. Compared to those used in previous studies (16, 17), our extraction conditions increased the extracted amounts of MKA by 22.81%, MKL by 11.34%, MS by 17.38%, AK by 18.56%, and CT by 340.55% (data not shown). In the extraction solvent experiment, 75% ethanol was found to be better than 95% ethanol and methanol, which were identified as ideal in the previous studies (16, 17). Further, we found that the extraction limitation of CT depended on an increase in the extraction temperature from 30 to 80°C; the extraction solvent was volatile at 90°C. In the extraction time experiment, we found that CT extraction was the rate-determining step in the extraction

procedure, and the extraction rate did not increase beyond 30 min. In the sample-solvent ratio experiment, we found that a dilution factor of 10 was the optimum, and it enabled better mixing of the RMR powder in the extraction solvent. Through this synchronous analytical method and optimal extraction procedure, we could obtain better analyses of the functional compounds and toxic compound from the RMR.

Based on previous studies, it was implied that azaphilone-like compounds might play roles in the regulation of cholesterol. The *Monascus* yellow pigments MS and AK are azaphilone-like compounds, and therefore, might exhibit some hypolipidemic potential. Tomoda et al. (21) reported that azaphilones exhibit structure-specific inhibition of cholesteryl ester transfer protein (CETP). CETP promotes the exchange and transfer of neutral lipids such as cholesteryl ester and triacylglycerol between plasma lipoproteins. Evidence has shown that CETP could decrease the level of low-density lipoprotein cholesterol and increase that of high-density lipoprotein cholesterol (21). In the future, we could investigate the effects of MK and azaphilone-like compounds on the regulation of cholesterol simultaneously with the multiplex analysis established in this study.

Conclusions

The use of 75% ethanol (10 mL) at 80°C for 30 min enables the extraction of the maximum amounts of MKA, MKL, MS, AK, and CT from 1 g RMR powder. These extraction conditions yielded an average recovery rate of 92.99, 95.61, 95.91, 92.63, and 91.12% for MKA, MKL, MS, AK, and CT, respectively. With regard to the optimal conditions for the HPLC, the peaks of MKA, MKL, MS, AK, and CT could be separated from noise peaks by the use of 0.05% TFA in acetonitrile–water (62.5 + 37.5, v/v) as the mobile phase for isocratic elution. In addition, the total time required for analysis, from RMR extraction to data calculation, was less than 90 min. Our synchronous analytical method is a precise and rapid method for simultaneously detecting the MKA, MKL, MS, AK, and CT levels in an RMR sample.

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References

(1) Albert, A.W., Chen, J., & Springer, J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3957–3961
(2) Endo, A. (1979) *J. Antibiot.* **32**, 852–854
(3) Endo, A. (1985) *J. Med. Chem.* **28**, 401–405
(4) Martinokova, L., Juzlova, P., & Vesely, D. (1995) *J. Appl. Bacteriol.* **79**, 609–616
(5) Tsuji, K., Ichikawa, T., Tanabe, N., Obata, H., Abe, S., Tarui, S., & Nakagawa, Y. (1992) *Nippon Shokuhin Kogyo Gakkaishi* **39**, 790–795
(6) Wu, C.L., Lee, C.L., & Pan, T.M. (2009) *J. Agric. Food Chem*. **57**, 5035–5041
(7) Lee, C.L., Wang, J.J., Kuo, S.L., & Pan, T.M. (2006) *Appl. Microbiol. Biotechnol.* **72**, 1254–1262
(8) Blanc, P.J., Laussac, J.P., Le Bars, J., Le Bars, P., Loret, M.O., Pareilleux, A., Prome, D., Prome, J.C., Santerre, A.L., & Goma, G. (1995) *Int. J. Food Microbiol.* **27**, 201–213
(9) International Agency for Research on Cancer (1986) *IARC,* Lyon, France, 83–98
(10) Frank, H.K. (1992) *Z. Erna-hungswiss.* **31**, 164–177
(11) Ribeiro, S.M., Chagas, G.M., Campello, A.P., & Kluppel, M.L. (1997) *Cell Biochem. Funct.* **15**, 203–209
(12) Akihisa, T., Tokuda, H., Ukiya, M., Kiyota, A., Yasukawa, K., Sakamoto, N., Kimura, Y., Suzuki, T., Takayasu, J., & Nishino, H. (2005) *Chem. Biodiversity* **2**, 1305–1309
(13) Su, N.W., Lin, Y.L., Lee, M.H., & Ho, C.Y. (2005) *J. Agric. Food Chem.* **53**, 1949–1954
(14) Martínková, L., Patáková-Jjzlová, P., Krent, V., Kucerová, Z., Havlícek, V., Olsovský, P., Hovorka, O., Ríhová, B., Veselý, D., Veselá, D., Ulrichová, J., & Prikrylová, V. (1999) *Food Addit. Contam.* **16**, 15–24
(15) Ho, B.Y., & Pan, T.M. (2009) *J. Agric. Food Chem.* **57**, 8258–8265
(16) Lee, C.L., Wang, J.J., & Pan, T.M. (2006) *J. AOAC Int.* **89**, 669–677
(17) Teng, S.S., & Feldheim, W. (1998) *Chromatographia* **47**, 529–536
(18) Akihisa, T., Tokuda, H., Yasukawa, K., Ukiya, M., Kiyota, A., Sakamoto, N., Suzuki, T., Tanabe, N., & Nishino, H. (2005) *J. Agric. Food Chem.* **53**, 562–565
(19) Wang, U.L., Houng, J.Y., Chang, H.S., Chien, H.C.R., & Hsu, W.H. (1998) *J. Chinese Agric. Chem. Soc.* **36**, 192–200
(20) Kycko, S., Shiho, S.S., Yoko, K., & Tamio, M. (1998) *Jpn. J. Food Chem.* **5**, 64–68
(21) Tomoda, H., Matsushima, C., Tabata, N., Namatame, I., Tanaka, H., Bamberger, M.J., Arai, H., Fukazawa, M., Inoue, K., & Omura, S. (1999) *J. Antibiot.* **52**, 160–170