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Development of a Rapid LC/DAD/FLD/MS^n Method for the Simultaneous Determination of Monacolins and Citrinin in Red Fermented Rice Products

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Supporting Information

ABSTRACT: Red fermented rice is used worldwide by many patients as an alternative therapy for hyperlipidemia; however, the discovery of a toxic fermentation byproduct, citrinin, causes much controversy about the safety of red mold rice products. A new and fast high-performance liquid chromatography method was developed and validated for simultaneous determination of cholesterol-lowering compounds monacolin K (lovastatin), monacolin K hydroxy acid, and other monacolins present in red fermented rice as well as nephrotoxic mycotoxin citrinin in a single run using connected diode array and fluorescence and mass spectrometric detectors. The proposed method was successfully applied for the analysis of red fermented rice food samples and various dietary supplements also containing other natural lipid-lowering agents. The deviations between label content and levels of active compounds found in investigated samples as well as high batch-to-batch variation found in one product indicate that the regular quality control of red fermented rice products is of great importance.

KEYWORDS: *red fermented rice, lovastatin, monacolins, citrinin, dietary supplement*

INTRODUCTION

Lovastatin (**1**) belongs to a class of lipid-lowering drugs called statins, which competitively inhibit the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in the cholesterol biosynthetic pathway. It is isolated as a secondary metabolite of the fermentation process of various fungi such as *Aspergillus terreus*,[1] *Monascus ruber*,[2] and *Penicillium* species.[3] The chemical structure of **1** contains a β-hydroxy-lactone group (Figure 1). The physiologically active form of the drug is the β-hydroxy acid (**2**), which is formed by a ring-opening reaction of the lactone ring. The prodrug lactone form can also exist in aqueous solution with its hydroxy acid equilibrium product, and this interconversion is highly pH dependent.

Although statins have been available for decades, many patients seek alternative therapies and effective ways to control cholesterol levels. An increased level of cholesterol is a primary risk factor for developing cardiovascular disease, the leading cause of death in the world. Red mold rice and its formulated products are currently used worldwide as a food or dietary supplement that have the ability of reducing cholesterol levels as efficiently as statin drugs.[4] Red mold rice is produced from the fermentation of rice (*Oryza sativa* L.) substrates by *Monascus purpureus*. It has been used in Chinese medicine for thousands of years to improve blood circulation and digestion. The main active ingredient of red mold rice contributing to the lipid-lowering effect is **1**, also known as monacolin K.[5] An earlier report showed that in fermented red rice, it is accompanied by 13 other bioactive statinlike compounds called monacolins.[6] However, **1** and **2** are the main active components that contribute to up to 90% of the total quantity of monacolins in the red mold rice. Therefore, their levels are taken as a label claim of some commercial red mold rice-related products.

Some *Monascus* strains could produce citrinin (**3**) as a secondary toxic metabolite that was previously found mainly in *Aspergillus* and *Penicillium* genera.[7] It (Figure 1) is a known mycotoxin, which causes functional and structural kidney damage as well as alterations in liver metabolism. The action of **3** at the cellular level is characterized by its interference with the electron transport system in mitochondria. Its discovery in *M. purpureus* fermented red rice causes much controversy about the safety of red mold rice products because up to 80% of them may contain this mycotoxin.[8] The maximum allowed level of **3** found as a contaminant in red fermented rice is 200 ppb in Japan, but the European Union has recommended a limit of 100 ppb.

The content of bioactive compounds in red rice dietary supplements is still not regulated. In addition, different *Monascus* strains showed variable **1** and **3** levels in red fermented rice.[9] Therefore, considerable variations were found in **1** and **3** content as well as the composition of monacolins in red mold rice products from different suppliers.[10] The discrepancies can also be found between claimed and actual contents of active ingredients. The increased number and complexity of red mold rice products, the absence of a transparent certification process, or internationally harmonized standards may result in the appearance of products on the market that lack acceptable quality and/or safety standards. On the other hand, red rice consumptions are rapidly growing because high cholesterol and cardiovascular diseases are being diagnosed more frequently. The quality control of red mold

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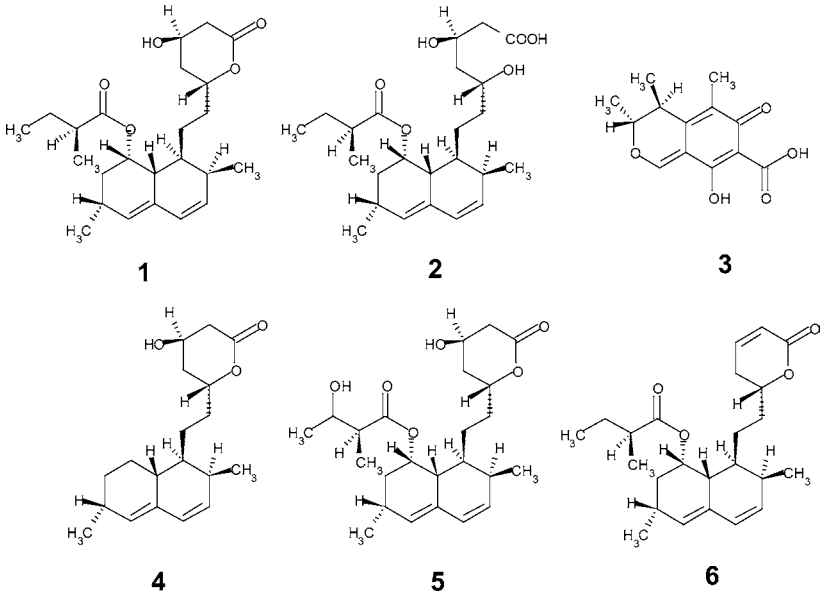

Figure 1. Chemical structures of monacolin K (lovastatin) (**1**), monacolin K hydroxy acid (**2**), citrinin (**3**), monacolin L (**4**), monacolin M (**5**), and dehydromonacolin K (**6**).

rice products and the determination of **1**, **2**, and total monacolins in these supplements have great significance for clinical therapy controls since their level is associated with cholesterol-lowering efficacy in vivo.

A number of methods are available for the determination of **1** in biological fluids and pharmaceutical dosage forms using liquid chromatography,[11,12] capillary electrophoresis,[13,14] UV spectroscopy,[15] and voltammety,[16,17] but the literature search has revealed that several high-performance liquid chromatography (HPLC) methods with diode array detection (DAD) combined with mass spectrometry were reported for the determination of monacolins in red mold rice and its formulated products.[4,10,18−20] In these methods, the analytical run time per sample took about 20−40 min. Very recently, fast screening of **1** in red rice products using flow injection tandem mass spectrometry was described.[21] This method takes under 1 min per analysis, but it is effective only in semiquantitative measurements.

Furthermore, the literature review revealed that **1** and **3** are usually determined by different analytical methods. The HPLC methods were developed for the determination of **1** in red mold rice products using a DAD detector, while **3** was quantitated with an indirect competitive ELISA,[22] a TLC technique,[23] or another HPLC method with a mass spectrometric detector.[10,24] Only two methods were described in the literature for the simultaneous quantitation of **1**, **2**, and **3**. Three analytes were separated and determined in red mold rice by a HPLC method with simultaneous UV detection used for **1** and **2** and fluorescence detection for the analysis of **3**.[25] The same authors developed a very similar method, including only a small change in the composition of mobile phase, for the simultaneous quantitation of **1**, **2**, **3**, and yellow pigments monascin and ankaflavin.[26] However, in both methods, the content of other monacolins in red mold rice dietary supplements was not determined.

No attempt has been made to date to determine **1**, **2**, and **3** as well as the total quantity of other monacolins simultaneously in red fermented rice and its formulated products by a universal HPLC method. The analytical method should be designed for the simultaneous quantitation of both lactone and hydroxy acid forms that in addition can potentially undergo interconversion. The monacolin K amount in red mold rice should include the concentration of lactone and hydroxy acid forms because both of them contribute to the cholesterol-lowering effects. Therefore, the aim of the present work is to develop a new universal reverse phase HPLC method for the rapid determination of **1**, **2**, other monacolins present in red fermented rice, and toxic fermentation byproduct, **3** (Figure 1), in a single run using connected DAD and fluorescence and mass detectors with the goal of establishing a quality control system to evaluate the efficiency and safety of red rice formulated products sold worldwide to the public. The method has been validated and successfully applied for the analysis of cholesterol-lowering agents in rice mold rice powder and various commercial available formulations of *Monascus* fermented red rice as well as safety evaluation regarding the presence of frequently present mycotoxin.

MATERIALS AND METHODS

Reagents and Chemicals. Standard **1** was kindly donated by Pliva (Zagreb, Croatia), while **3** was supplied from Sigma-Aldrich (Steinheim, Germany). Methanol, ethanol, acetonitrile, and formic acid, HPLC grade, were purchased from Merck (Darmstadt, Germany). The ultrapure water was prepared with a Mili-Q water purification system (Milipore, Bedford, MA).

Preparation of Stock and Working Solutions. A stock standard solution (1 mg/mL) of **1** was prepared using acetonitrile and Milli-Q water (1:1, v/v), while a stock solution of **3** (0.2 mg/mL) was prepared by dissolving with methanol. A stock standard solution of **2** was prepared by the hydrolysis of the lactone form under the optimized conditions described earlier[27,28] by the addition of 0.1 M NaOH solution in acetonitrile:water (1:1, v/v) to a stock solution of **1** in an equal amount. To complete the hydrolysis of the lactone form in alkaline solution, the samples were left for 1 h at 45 °C in an ultrasonic bath. The realization of conversion of lactone to its acidic form was confirmed by LC/MS analysis. The analysis confirmed that the lactone form was completely converted to the open ring hydroxy acid form (more than 99.9%). All stock solutions were stored in a refrigerator at 4 °C, and working solutions were prepared daily by serial dilutions with the mobile phase just before measurements.

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Table 1. List of Investigated Red Mold Rice Products

product name	manufacturer	product type	description	sample type
Red mold rice	Bangkok, Thailand	food	organically grown red mold rice	grain
Thailand red jasmine rice	Bangkok, Thailand	food	organically grown red mold rice	grain
Red rice	Donji Stupnik, Croatia	dietary supplement	red mold rice containing at least 1.5% monacolin K	capsule
Cholesterol maintenance	Park City, United States	dietary supplement	dietary supplement containing 0.6 g of red mold rice, *Commiphora mukul* gum extract, *Cynara scolymus* leaf extract, L-arginine, non-GMO soy bean concentrate, γ-oryzanol, black pepper extract, ginger root extract, rosemary leaf extract, turmeric root extract, and cayenne extract	tablet
No-Colest Omegasol	Bussolengo, Italy	dietary supplement	dietary supplement with declared amount of 200 mg of red fermented rice (standardized to 1.5% monacolins), *Citrus bergamia*, fatty acids, GMO free soy lecitin, and chlorofil	softgel
Rizolip	Ludbreg, Croatia	dietary supplement	red mold rice containing 1% monacolin K	capsule
Omelip	Ludbreg, Croatia	dietary supplement	dietary supplement containing 200 mg of dried red mold rice extract containing 1.5% monacolin K, 1000 mg of fish oil (18% EPA, 12% DHA), vitamin C, and vitamin E	liquid capsule
Normolip 5	Albissola Marina, Italy	dietary supplement	dietary supplement containing 100 mg of red fermented rice standardized to 3% monacolins, 150 mg of γ-oryzanol, 120 mg of phytosterols, and 5 mg of policozanol	capsule

Samples and Sample Preparation. Two products of red fermented rice were purchased from a local health food store, and six dietary supplements containing red fermented rice were supplied from a community pharmacy. The dietary supplements analyzed in this work were in multiple dosage forms including tablets, capsules, liquid capsules, and softgel. Most of the products contained only red fermented rice as a natural lipid-lowering ingredient. A few other products contained additional ingredients. A list and description of products together with their manufacturers are given in Table 1.

The grains of red fermented rice were ground to a fine powder using a mortar and pestle. Ten tablets were weighed, and the average of one tablet was determined. Afterward, all tablets were finely ground and used in further investigations. Likewise, the content of 10 capsules, liquid capsules, or softgels was pooled, and the average weight of one capsule or softgel was calculated. About 1 g of each homogenized sample, accurately weighed, was transfer to a 15 mL centrifuge tube. The suspensions were extracted with 10.0 mL of 80% methanol for 60 min at room temperature using an ultrasonic bath. Finally, the solution was cleared by centrifugation at 3000*g* for 10 min at 25 °C, and the supernatant was collected and then filtered through a 0.45 μm Chromafil membrane filter (Macherey-Nagel, Düren, Germany) before injection.

LC/DAD/FLD/MS^n Conditions. The liquid chromatographic system was an Agilent Series 1100 HPLC (Agilent Technologies, Waldbronn, Germany) comprised of the following modular components: a vacuum degassing unit, a quaternary pump, an automatic sample injector with a 100 well tray, a column oven, a diode array, and a florescence detector. The different columns were examined: Zorbax SB-C18, 250 mm × 4.6 mm, 5 μm, Hypersil, 150 mm × 4.6 mm, 5 μm (Agilent Technologies, Waldbronn, Germany), Symmetry C18, 150 mm × 4.6 mm, 3.5 μm and XBridge C18, 50 mm × 3.0 mm, 2.5 μm (Waters, Milford, MA). The mobile phase consisted of (A) acetonitrile, water, and formic acid (10:90:0.1, v/v/v) and (B) acetonitrile, water, and formic acid (90:10:0.05, v/v/v). Both mobile phases were filtered through a cellulose nitrate filter, diameter 47 mm, pore size 0.45 μm (Sartorius, Goettingen, Germany). The binary gradient program was as follows: 40−70% B (0−7 min) and 70−90% B (7−10 min). After the gradient separation, the column was re-equilibrated for 5 min using the initial solvent composition. The flow rate was set to 1 mL/min, and split (1:2) to MS detector was used. The samples were kept in amber vials at 4 °C in the autosampler, and the injected volume was 5 μL. The separation was performed at 25.0 ± 0.1 °C.

The UV detection and quantitation of compounds were performed at 237 nm, and UV spectra were recorded within a range of 200−400 nm. The excitation and emission wavelengths for fluorimetric detection of **3** were set at 331 and 500 nm, respectively.

The mass detection was conducted using an Agilent MSD Trap system equipped with an electrospray ion source and an ion trap analyzer system. To maximize method sensitivity, the electrospray source temperature was optimized at 350 °C, and the capillary voltage was set at 3.5 kV. Nitrogen was used both as a drying gas at a flow fate of 10 L/min and as a nebulizer gas at a pressure of 20 psi. The full scan mass spectra were acquired over a range of m/z 100−600. Helium was used as the collision gas, and the MS^n studies were carried out by keeping the collision energy at 30%. Data acquisition and processing were done using ChemStation for LC 3D and LC/MSD Trap v.5.2 software.

Validation. Method validation was performed following the recommendation of International Conference on Harmonization. The method was validated for selectivity, linearity, extraction efficacy, precision, and accuracy. The limits of determination (LOD) and quantitation (LOQ) as well as the stability of analytes in samples and standard solutions were also tested. [29]

RESULTS AND DISCUSSION

Method Development. The investigated analytes have fairly different physicochemical properties. The lipophilicity of **1** calculated by ALOGPS v 2.1 program was 4.11; for **2**, the predicted value was 3.74, while it was 1.23 for the most hydrophilic analyte, **3**.[30,31] Moreover, the aim of our work was to identify other monacolins usually present in red fermented rice. Although it was found that all monacolins are inhibitors of enzyme HMG-CoA reductase, they have different chemical structures and therefore different physicochemical properties (Figure 1). Consequently, finding a suitable sample preparation procedure and chromatographic conditions was a quite challenging task as several compounds with different properties were investigated. Furthermore, different sample types (grains/tablets/capsules/softgels) containing red fermented rice as well as other natural cholesterol-lowering remedies together with different excipients were analyzed.

To optimize the extraction process, a representative specimen of each sample type was used to get better insight into the extraction process and the influence of other sample constituents. First, the organic solvents of different polarities (methanol, ethanol, and acetonitrile) were used to assess their extraction efficiency. As the best recoveries were obtained using methanol, further investigations were performed using methanol with different proportions of ultrapure water (50, 40, 30, 20, and 10%). The use of 80% methanol as an extraction solvent the maximum amount of all target compounds was obtained. In the work of Huang and co-workers,[32] it was reported that after extraction with methanol, **2** can react with

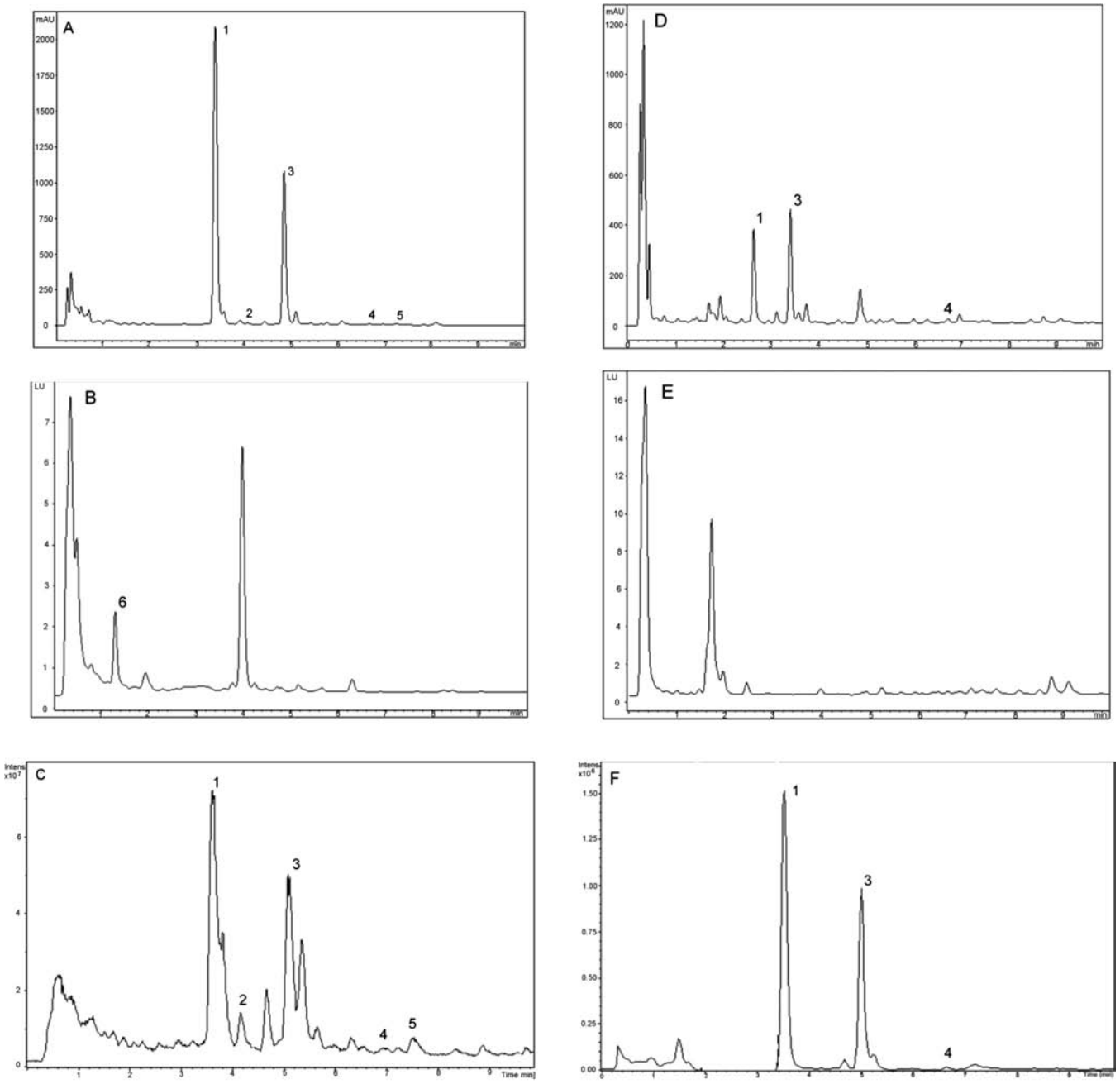

Figure 2. Representative chromatograms: (A) LC/DAD, (B) LC/FLD, (C) TIC chromatograms of dietary supplement containing only red fermented rice, (D) LC/DAD, (E) LC/FLD, and (F) TIC chromatograms of dietary supplement containing red fermented rice with other natural lipid-lowering agents. Peaks: 1, monacolin K hydroxy acid; 2, monacolin L; 3, monacolin K (lovastatin); 4, monacolin M; 5, dehydromonacolin K; and 6, citrinin.

methanol to form an ester. The published results showed that the concentration of methanol and the storage time affected the transformation of **2** to the methyl ester form when extractions were performed using methanol and water mixtures in acidic conditions. Therefore, mass spectrometry was used to identify extracted analytes from red mold rice products using aqueous methanol solutions. Our results showed that the esterification of **1** and **2** did not occur using methanol/water mixtures as the extraction solvent. As an extraction procedure should yield the highest amount of analytes in the shortest time, the influence of extraction time in a range from 15 to 90 min on the extraction efficacy was investigated. The maximum amount of each analyte was extracted from all sample types at 60 min, and a further increase of extraction time did not increase the extraction efficacy. Heat is usually used to accelerate the extraction process and to obtain the higher levels of analytes. Although several authors have reported the thermal degradation of **3** at high temperatures, the influence of temperature on the extraction efficacy of analytes was investigated.[33] The extraction efficacy of monacolins was not significantly improved at high temperatures. Therefore, to avoid the degradation of **3**, an extraction procedure was performed only at room temperature.

Various columns and mobile phase compositions were tested to find the optimal chromatographic conditions. Each column provided a different combination of hydrophobicity, silanol activity, hydrolytic stability, and interaction with analytes. The

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Table 2. Monacolins and Citrinin Content in Investigated Food and Dietary Supplement Samples Analyzed by the Developed LC/DAD/FLD/MS^n Method

	compound								
	1 (μg/g)[a]	**2** (μg/g)[a]	**3** (ppb)[a]	**4** (μg/g)[a,b]	**5** (μg/g)[a,b]	**6** (μg/g)[a,b]	monacolins (μg/g)[c]	amounts of monacolins per recommended daily serving (mg)	detected/labeled (%)[d]
					food samples				
1	<LOQ	25.1	ND[e]	ND	ND	ND	25.1	2.01	NL[f]
2	4.4	15.8	ND	ND	ND	ND	20.2	1.62	NL
					dietary supplement samples				
1	3760	7644	95	ND	6	13	11423	11.42	76.2
2	8056	513	98	239	246	2792	11846	11.85	118.7
3	6276	228	ND	21	24	414	6963	3.48	116.1
4	510	1351	ND	ND	12	ND	1873	6.37	NL
5	6950	2236	ND	85	<LOQ	25	9296	4.65	154.9
6	ND	47	ND	ND	ND	ND	47	0.05	2.0

[a]Mean of three determinations. [b]Contents of **4**–**6** were calculated using the regression equation obtained for **1**. [c]Total quantity of all monacolins. [d]Detected monacolins contents/labeled value (%). [e]ND, not detected. [f]NL, not labeled.

shortest analysis time still with good resolution and peak shapes without tailing was observed using XBridge C18 column.

The interconversion between **1** and its acid form is known to be taking place by its hydrolysis. This phenomenon may cause increased variance in the accuracy of the method and depends on several factors such as storage conditions, pH, and temperature. As it was already reported, the pH of mobile phase considerably influences the interconversion procedure, with pH 4.5 being the optimal value for the minimum interconversion rate.[27,28] Therefore, the specific pH was applied for chromatographic conditions. To obtain a good separation and resolution of all analytes, various isocratic solvent systems were used. As the total analysis time was too long, gradient elution was employed. The effect of column temperature on the separation of analytes in the range from 25 to 35 °C was investigated, but increasing the temperature above 25 °C resulted in the rapid elution of **3** close to the solvent front. Initially, UV detection was applied for the determination of all analytes, but anticipated levels of **3** in selected samples were too low (below 100 ppb) for its quantitation by UV detection. Therefore, fluorescence detection was adopted due to its high selectivity and sensitivity.

The representative chromatograms of two various dietary supplements are presented in Figure 2. The retention times of **3**, **2**, and **1** were 1.25 ± 0.01, 3.45 ± 0.01, and 4.99 ± 0.01 min (n = 6), respectively. The optimized conditions yielded symmetrical and sharp peaks of all three analytes with peak purities higher than 999.1. Furthermore, the obtained chromatograms reveal that in spite of a complex matrix of the samples, almost no other components were coeluted with compounds of interest. Moreover, all other constituents of samples were eluted in 10 min, allowing their further structural characterization and identification by tandem mass spectrometry.

Method Validation. *Extraction Efficacy.* The extraction efficacy and reproducibility of the extraction procedure for **1** (5 μg/mL), **2** (5 μg/mL), and **3** (1 μg/mL) were determined in each representative sample type by comparing the responses from samples spiked before extraction with those from samples extracted and spiked after extraction (Table S1 in the Supporting Information). All extraction recoveries were relatively high (98–103%), which can be explained by the simple and effective sample preparation procedure. Also, it should be pointed out that, despite the wide variability of matrix type and composition, adequate extraction recoveries for all three analytes were achieved in all sample types.

Linearity, LOD, and LOQ. The linearity of **1** and **2** was tested in the range from 1 to 500 μg/mL, while the linearity of **3** was tested in the range from 0.001 to 10 μg/mL (Table S2 in the Supporting Information). At least nine concentration levels were used in all calibration curves. The obtained correlation coefficients were higher than 0.999, indicating satisfactory linearity of the developed method. The LODs and LOQs were determined by injecting a series of dilute solutions with known concentrations. LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10, respectively. The LOQs using UV detection were quite low, 0.3 and 0.2 μg/mL for **1** and **2**, respectively. It should be pointed out that LOD and LOQ values obtained for **3** using fluorimetric detection were even lower, 0.0005 and 0.001 μg/mL, indicating good sensitivity of the method.

Precision. Method precision experiments were performed using spiked assays of each representative sample type (5 μg/mL for **1** and **2**; 1 μg/mL for **3**). The sample preparation procedure and analysis were repeated six times within the same day to obtain the intraday precision, while the interday precision was assessed by three replicate analyses on three consecutive days. The results of precision measurements, expressed as relative standard deviations (RSDs), are listed in Table S3 in the Supporting Information. The RSD values for intra- and interday precision were lower than 1.6 and 3.8%, respectively. The obtained data revealed that the proposed method was reproducible.

Accuracy. Accuracy was assessed by the determination of recovery using the standard addition method. Samples were prepared by spiking each representative sample type with three different levels of all analytes, and the procedure was repeated three times. The recoveries were calculated based on the ratio of added and obtained amounts. The results are summarized in Table S4 in the Supporting Information. The data showed the satisfactory accuracy of the proposed method as recoveries of all analytes were in the range from 98 to 104%. As expected, improved values were observed at higher concentrations of analytes.

Stability. As part of the method validation, data were also generated to ensure that all analytes were stable at distinct timing and temperature conditions. The stability tests were performed in terms of short-term and long-term storage and

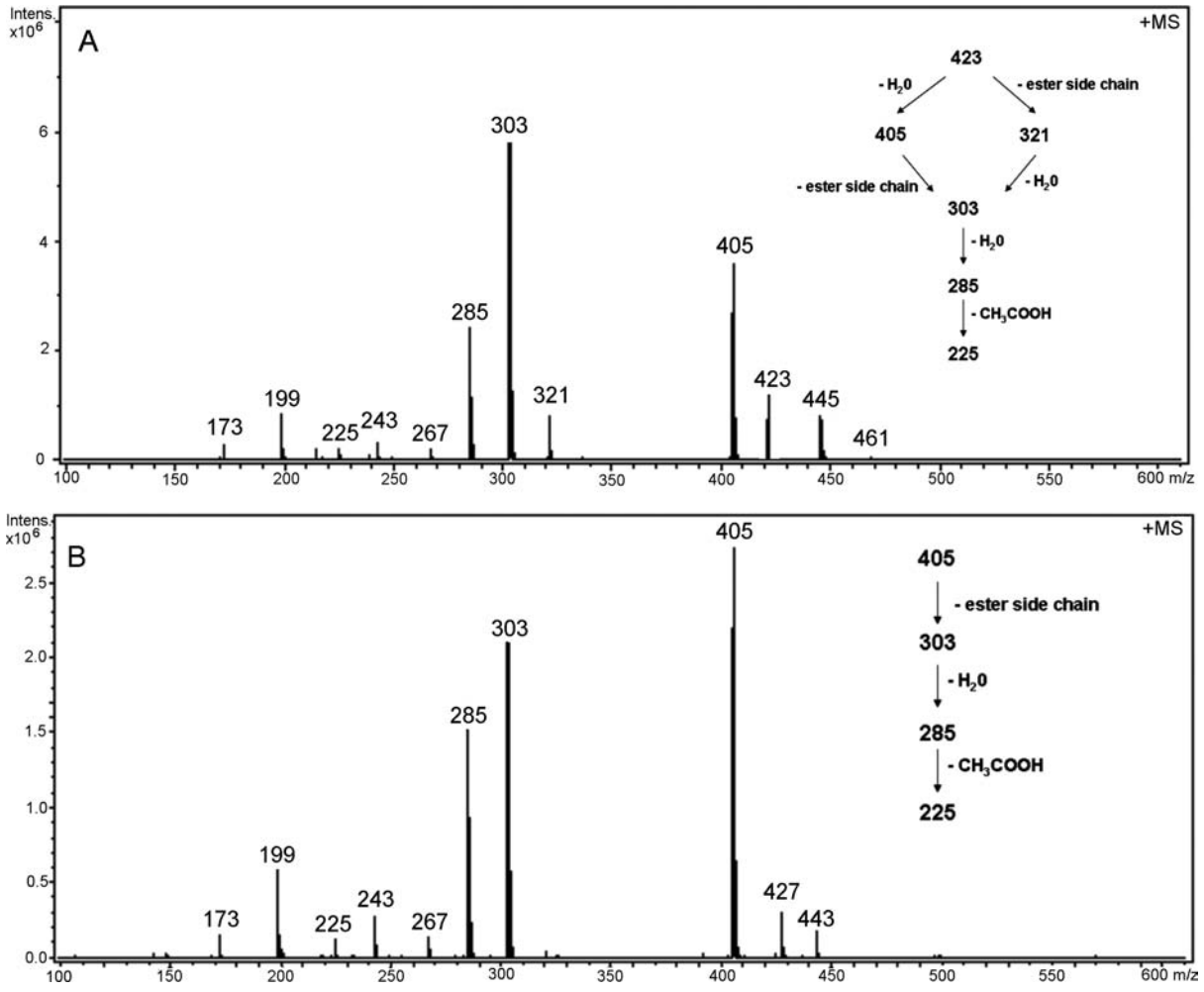

Figure 3. Mass spectra of (A) monacolin K acid and (B) monacolin K and their proposed fragmentation pathways to the ion at m/z 225.

autosampler stability. Moreover, the stability of analytes in the spiked representative samples as well as in the standard solutions was analyzed. The short-term (room temperature) stability was assessed at room temperature for 6 h as it was a period of time exceeding that expected to be encountered during the routine sample preparation. The long-term stability was assesed at −20 °C for 10 days. The autosampler stability was tested by storing the samples at 4 °C into an autosampler for 24 h. The percentage of analytes recovered from samples ranged from 98.3 to 99.4%. These results indicate that degradation of all analytes was not significant during the chosen conditions.

Analysis of Food and Dietary Supplement Samples. The usefulness of the proposed method was evaluated by determination of all three analytes in red mold rice food and dietary supplement samples supplied from local health food stores and community pharmacies. The levels of all analytes were summarized in Table 2. As was expected, significantly lower amounts of **1** and **2** were found in food samples than in dietary supplements. Although in some Asian countries, where red mold rice is a common food additive, a person's normal daily intake might go as high as 80 g, the amount of monacolins taken by food is still quite low. However, there was considerable variability of **1** and **2** amounts in the investigated dietary supplements. Although it was expected that the dominant compound in all investigated dietary supplement would be **1**, in two samples, the amount of the acid form was significantly higher than the lactone form. Moreover, in one sample, only the acid form was detected. As was anticipated, lower amounts of both monacolins were found in dietary supplements consisting of several natural hypolipidemics.

The previous studies[7,9] have demonstrated the presence of **3**, a highly toxic mycotoxin produced by several *Monascus* species, in poorly manufactured red mold rice products. Therefore, the aim of this work also was to determine its levels present in investigated red mold rice food and dietary supplements samples. The above-described method allowed quantitation of quite low levels of **3** (10 ppb). It was not detected in food samples, while it was found in two samples of dietary supplements (Table 2). However, none of the products exceeded the European Union contamination level for **3** (100 ppb). According to our results, investigated red mold rice food and dietary supplement products are reliable for human use with regard to contamination of this mycotoxin.

In several samples, four peaks with the same UV spectrum of mountain-like peak at λ_{max} 237 nm as **1** gave were found. To clarify whether these compounds belong to group of monacolins, hyphenated instrumentation of HPLC with tandem mass spectrometry using a developed LC method was applied. To get an insight into the fragmentation pattern of monacolins, MS and MS^2 spectra of **1** and **2** were studied (Figure 3). Under the above-described ESI-MS conditions, **1**

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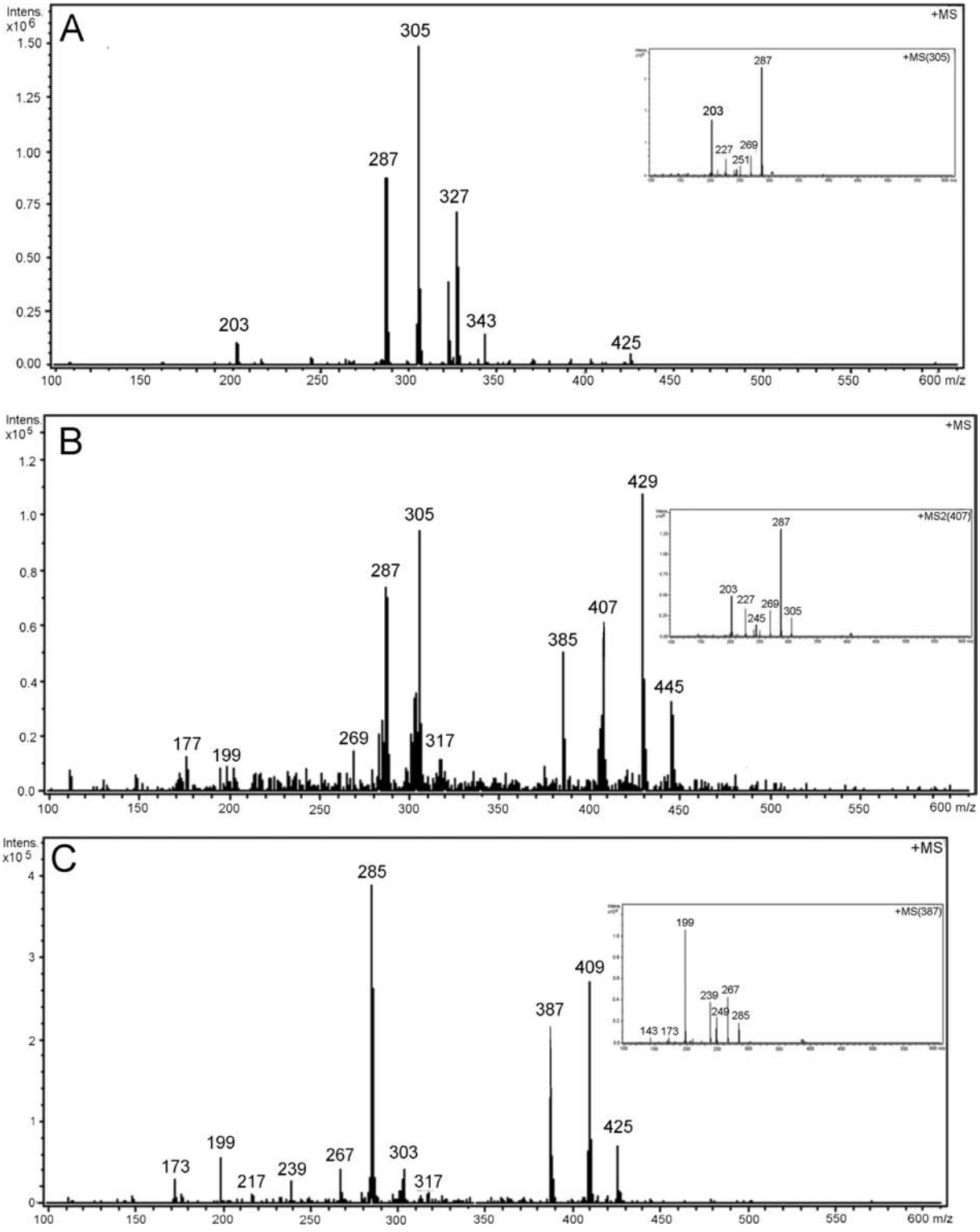

Figure 4. ESI-MS spectra of (A) monacolin L, (B) monacolin M, and (C) dehydromonacolin K (inserts: ESI-MS^2 spectra of each pseudomolecular ion).

produced abundant ions corresponding to the protonated molecule (M + H) at m/z 405 as well as sodium and potassium ion adducts at m/z 427 and 443, respectively. The pseudomolecular ion at m/z 405 generated complex MS^2 spectra with the formation of multiple fragment ions. The major fragment ions at m/z 303, 285, 243, and 199 were detected. Minor fragment ions including m/z 267 and 225 were also observed. The ESI-MS spectra of **2** clearly showed the characteristic diagnostic ions at m/z 423 (M + H), 445 (M + Na), and 461 (M + K). The ESI-MS^2 spectra of the pseudomolecular ion at m/z 423 indicated the presence of the product ions with m/z at 405, 321, 303, 285, 264, 243, 225, and 199. These results suggest that the major fragments were obtained by loss of a water molecule and/or the ester side

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chain. Fragmentation of both compounds generated an identical ion at m/z 303 formed after the loss of the ester side chain. Furthermore, the formation of the ion at m/z 285 was generated by the neutral loss of a water molecule from the ion at m/z 303. The further fragmentation of this ion proceeded in the same fashion.

The peak at retention time 4.10 min (Figure 2C) displayed the pseudomolecular ion at 305 as well as sodium and potassium adduct ions at m/z 327 and 343, respectively (Figure 4A). Afterward, the ion at m/z 287 was generated by the neutral loss of a water molecule from the pseudomolecular ion at m/z 305. According to the literature and mass spectrometry data, this compound may be assumed to be monacolin L (**4**).[6] The ESI-MS spectra of the peak at t_R 6.62 min showed the pseudomolecular ion at m/z 407 and the adduct ions at m/z 429 and 445 (Figure 4B). The MS^2 spectra of the pseudomolecular ion showed the presence of the fragment ions at m/z 305 and 287 corresponding to loss of the ester side chain and a water molecule. According to literature data, MS, and MS^2 spectra, it may be concluded that this peak belongs to monacolin M (**5**). The mass fragmentation pattern of peak at t_R 7.29 min was largely similar to the other identified monacolins (Figure 4C). The major ions at m/z 387, 409, and 425 belong to pseudomolecular ion, sodium, and potassium adduct ions, respectively. Furthermore, the ion at m/z 387 yielded m/z 285 and 267 by the losses of the ester side chain and dehydration. The obtained spectra indicate that dehydromonacolin K (**6**) was present in even four investigated samples.

In Table 2, the total amount of all monacolins are summarized. In red mold rice food samples, none of other monacolins were found, while in most dietary supplements, other monacolins beside **1** and **2** were present. Levels of the other monacolins in investigated supplements were quite low, except in one sample (3277 μg/g). Table 2 also summarizes the daily amounts of monacolins that patients would ingest if they follow the daily serving recommendation by the producer. Obviously, quite different amounts of monacolins could be taken using different red mold rice dietary supplements. In addition, obtained levels of **1** and **2** were compared with values marked on the product labels. It should be noted that not all samples have a standardized level of monacolins. Moreover, the dramatic variation from labeled values was found (Table 2). In one sample, only 2.0% of declared amount was determined, while in another, the amount of monacolins was far above labeled value (154.9%). According to obtained results, it is possible to conclude that red mold rice dietary supplements are poorly standardized, resulting in inconsistent composition and biological activity.

The type of *Monascus* strain, fermentation temperature, incubation period, composition, and pH of medium solution can significantly affect the composition of monacolins in red mold rice, which can lead to variable, unpredictable, low, or even toxic levels of active ingredients.[22] Therefore, it is necessary to evaluate the batch-to-batch variability of red mold rice products. Three different batches of each food and dietary supplement sample were investigated. According to our results, a quite high batch-to-batch uniformity was found for most of products (RSDs were lower than 2.2%), while one dietary supplement exhibited a large batch-to-batch variation (RSD was 14.8%).

The deviations between label content and levels of active compounds found in investigated samples as well as high batch-to-batch variation found in one product indicate that the regular quality control of red fermented rice products used in the fields of preventive nutrition and alternative therapy for hyperlipidemia is of great importance. Therefore, we developed a new reverse phase HPLC method for the simultaneous determination of monacolins present in red fermented rice as well as toxic fermentation byproducts. Comparing the proposed method with other previously published methods for the simultaneous determination of **1**, **2**, and **3** (run time around 20 min),[25,26] it should be pointed out that it is much shorter and therefore more appropriate for routine control of red fermented rice products. It is believed that all monacolins contribute to the cholesterol-lowering effects of red fermented rice; thus, the proposed method is convenient even for their determination. Also, complicated as well as expensive sample preparation procedures were avoided. In addition, the method was successfully applied for the analysis of monacolins present in dietary supplements containing not only red fermented rice but also other cholesterol-lowering agents.

■ ASSOCIATED CONTENT

Supporting Information

Tables of extraction efficacy, method calibration, intra- and interassay precision, and accuracy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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