Characterization of Red Yeast Rice Products and Raw Materials Using Accurate-Mass Q-TOF LC/MS and Principal Component Analysis

Application Note

Food, Dietary Supplements

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Abstract

Rice fermented with the mold *Monascus purpureus*, also known as the red yeast rice (RYR) used in traditional Chinese medicine, contains pharmacologically important monacolins. The prescription statin, lovastatin, is identical to monacolin K, and there is a suspicion of spiking RYR products with lovastatin. Profiling authentic and raw RYR samples has the potential to distinguish the spiked from natural product. *Monascus purpureus* is also known to produce the mycotoxin citrinin. Sudan red G, considered possibly genotoxic or carcinogenic by the European Food Safety Authority (EFSA), is a RYR adulterant. Pigments produced by *Monascus spp*. are used as coloring agents. To address regulatory requirements, and assess product and raw material quality, origin, and safety, a robust method for characterizing and quantitating monacolins, pigments, and contaminants such as citrinin is needed. This application note presents a method that uses the Agilent Accurate-Mass Q-TOF LC/MS system and Agilent MassHunter Workstation software with principal component analysis (PCA) to characterize three authentic (AU) RYR samples, 31 RYR raw materials (CR), and 14 dietary supplements (DS). Quantitation was performed using UHPLC DAD. The method uncovered notable variation among RYR samples and raw materials. High mass accuracy Q-TOF LC/MS data enabled determination of compound identities, including identities of isobaric compounds. PCA showed that monacolin and pigment type could be used to classify RYR samples to determine the authenticity and quality of other RYR samples.

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Introduction

This application note presents a robust method that applies the Agilent Accurate-Mass Q-TOF LC/MS system and Agilent MassHunter Workstation software with principal component analysis (PCA) to the qualitative analysis of monacolins, pigments, citrinin, and sudan red G in red yeast rice (RYR) samples. PCA was used to distinguish among samples to explore its utility in determining RYR sample identity and quality. Quantitation was through UHPLC DAD. The complementary study "Chemical profiling and quantification of monacolins and citrinin in red yeast rice commercial raw materials and dietary supplements using liquid chromatography-accurate Q-TOF mass spectrometry: Chemometrics application" [1] provides a detailed description of the method, including method validation and complete analytical results.

Rice fermented with the mold *Monascus purpureus*, RYR, is used in traditional Chinese medicine to invigorate the body, aid in digestion, and revitalize the blood. RYR contains the pharmacologically important monacolins J, K (lovastatin), L, M, and X, as well as acid forms of J, K, L, M, and X, and dihydromonacolin K. Of these, monacolin K and its dihydro derivative are the most active [2]. Different strains of *Monascus purpureus* produce different amounts of monacolins.

In the US, the FDA regulates finished dietary supplement products and dietary ingredients per the Dietary Supplement Health and Education Act of 1994 (DSHEA). DSHEA specifies that dietary supplement manufacturers are responsible to ensure their products are safe before they are marketed, and that product label information is truthful and not misleading.

The safety of commercial RYR products has not been established. Their monacolin content has been shown to vary over a wide range, and is not generally listed on product labels [3]. In addition, spiking RYR products with lovastatin to enhance their activity is suspected. Lovastatin, which is identical to monacolin K, is a prescription statin used to treat high cholesterol. Profiling RYR samples for relative amounts of monacolins, including monacolin K (lovastatin), is highly desirable. *Monascus purpureus* is known to produce the mycotoxin citrinin, which has been found in RYR [3]. Sudan red G, a yellow-red lysochrome azo dye considered possibly genotoxic or carcinogenic by the European Food Safety Authority (EFSA) [4], can be a RYR adulterant [1].

Pigments produced by *Monascus spp*. are used in Asia as coloring agents to improve the appearance and organoleptic properties of foods. They are very stable compared to synthetic dyes. Pigments include the orange pigments monascorubin (MS) and rubropunctatin, the yellow pigments monascin and ankaflavin (AK), and the red pigments monascorubramine and rubropunctamine. The color produced is influenced by the culture conditions, such the pH and the phosphorus and nitrogen source in the substrate [5].

To address regulatory requirements, and to assess RYR product and raw material quality, origin, and safety, a robust method for profiling RYR samples is needed.

Experimental

Chemicals

Citrinin, lovastatin (monacolin K), sudan red G, mevastatin (compactin), lovastatin diol lactone (monacolin J), and dehydrolovastatin (dehydromonacolin K) were purchased from various sources. Monacolin K acid (lovastatin acid) was prepared by hydrolyzing lovastatin with a methanolic 0.1 N sodium hydroxide solution.

RYR Samples

Three RYR authentic (AU) samples were obtained from Beijing Peking University WBL Biotech Co., Ltd., China. Thirty-one samples of RYR raw materials (CR) frequently purchased by supplement manufacturers in USA and elsewhere were procured from China. Fourteen dietary supplements (DS) labeled as containing 600 or 1,200 mg of RYR were purchased online from supplement retailers in the USA.

Standards

Stock solutions of the standard compounds were prepared at a concentration of 2 mg/mL in methanol. Calibration curves were at seven concentration levels. The concentration levels were 0.5–100 µg/mL for citrinin and 0.3–500 µg/mL for the other compounds measured using UHPLC DAD.

Sample preparation and extraction

Dietary supplement capsules/tablets

For tablets, five were weighed and pulverized with a mortar and pestle. For capsules, five were weighed, opened, and the contents mixed and triturated using a mortar and pestle.

Authentic samples, raw materials and dietary supplements
Dry samples (0.5 g) and capsules/tablets were weighed (average weight of dosage form), then sonicated in 2.5 mL of methanol for 30 minutes followed by centrifugation for 15 minutes at 959 × g. The supernatant was transferred to a 10-mL volumetric flask. This procedure was repeated four more times with 2.0 mL methanol, and the supernatants were combined. The final volume was adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection into the LC, 2 mL was passed through a 0.45-µm PTFE membrane filter and the first 1.0 mL was discarded. The concentrations of sample solutions were optimized to 50 mg/mL (a 1:10 dilution of the original 0.5 g).

Instrumentation

Analysis of reference compounds and RYR extracts were performed using a reversed-phase chromatographic system without consideration of any specific group of compounds. The samples were separated using an Agilent 1290 Infinity LC System equipped with a binary pump, vacuum solvent micro degasser, autosampler with 108-well tray, thermostatically controlled column compartment, and photo diode array detector. The solvent system was optimized to elute all detectable compounds within 20 minutes. UHPLC parameters are shown in Table 1.

Table 1. UHPLC Parameters

Instrument	Agilent 1290 Infinity LC System with DAD
Column	Agilent ZORBAX SB-C18 RRHD 2.1 × 150 mm, 1.8 µm (p/n 859700-902)
Mobile phases	A) Water with 0.1 % formic acid B) Acetonitrile with 0.1 % formic acid
Gradient	65 %A/35 %B to 35 %A/65 %B in 15 minutes, and in next 3 minutes to 100 %B
Flow rate	0.35 mL/min
Post run column equilibration	5 minutes
Column temperature	35 ˚C
Injection volume	2.0 µL

Mass spectrometry was performed using an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system equipped with an Agilent Jet Stream electrospray source and controlled by Agilent MassHunter Acquisition software. The Q-TOF mass spectrometer conditions were optimized to detect the maximum number of compounds including reference standards. Q-TOF mass spectrometer parameters are shown in Table 2.

Table 2. Agilent 6530 Q-TOF Mass Spectrometer Parameters

Instrument	Agilent 6530 Accurate-Mass Q-TOF LC/MS system
Ionization mode	Positive ion electrospray with Agilent Jet Stream technology
Instrument mode	2 GHz extended dynamic range
Mass range	100–1,000 *m/z*
Collision gas (CID)	Nitrogen
Drying gas (N_2)	250 °C at 9 L/min
Sheath gas	325 °C at 10 L/min
Nebulizer gas	35 psi
Fragmentor	125 V
Capillary	3,500 V
Nozzle	0 V
Skimmer	65 V
Octopole RF	750 V

Accurate mass measurements were obtained through reference ion correction using reference masses at *m/z* 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine, or HP-921] in positive ion mode. The reference solution was introduced into the ESI source through a T-junction using an Agilent 1200 Series Isocratic Pump using a 100:1 splitter set at a flow rate of 20 µL/min.

Single MS experiments were used for compound identification and feature extraction. The quadrupole was set to pass all ions into the TOF analyzer, where they were measured at 1 second per spectrum.

ESI-MS-MS collision induced dissociation (CID) experiments were used to identify compounds. Precursor ions of interest ($[M+H]^+$ ions) were selected by the quadrupole mass filter and then subjected to collision with nitrogen in a high-pressure collision cell. Major fragments were obtained for monacolins, pigments, citrinin, and sudan red G. These fragments were compared with data produced for the standards under the same instrumental conditions.

Data analysis

MassHunter Qualitative Analysis software was used to process the DAD data and raw MS and MS/MS data using "Find by Molecular Feature" (MF). To subtract MFs originating from the background, a methanol blank was analyzed using identical instrument settings. The measured blank (background) MFs were then removed by exporting them to a .csv file, and including the blank mass list in the mass filters (to exclude these mass(es)).

Background-subtracted data were converted into compound exchange format (.cef) files so they could be analyzed by Agilent Mass Profiler Professional (MPP) software. The .cef files included the retention time, absolute abundance, and mass of the molecule from the MF extraction. MPP software was used to perform the PCA of the RYR samples. For analysis by MPP software, the retention time and mass alignment across the sample sets was performed using a tolerance window of 0.2 minutes and 20 mDa.

Results and Discussion

Compound identification

Figure 1 shows the UHPLC DAD (237 nm) chromatogram and Q-TOF LC/MS positive ion ESI base peak chromatogram for two RYR samples: CR15674 (raw material) and AU15680 (authentic sample). Citrinin (1), monacolin J (lovastatin diol lactone) (2), monacolin K acid (3), compactin (mevastatin) (4), monacolin K (lovastatin) (5), and dehydromonacolin K (dehydrolovastatin) (6) peaks were found. Retention time variability across the samples was 3 to 4 seconds with a relative standard deviation of less than 2%.

Significant chemical differences were found among the RYR samples. Samples such as CR15674 had high amounts of pigments, and no monacolins. Other samples such as AU15680 were found to have high amounts of monacolins, and few pigments.

As shown in Figure 1, the 6530 Q-TOF LC/MS analysis produced significantly more chromatographic peaks than LC DAD, and could provide lower level quantification and uncover more compounds for identification. For food safely applications where mycotoxins such as citrinin are monitored at parts-per-billion (ppb) levels, the Agilent 6530 Q-TOF LC/MS method provides a clear advantage.

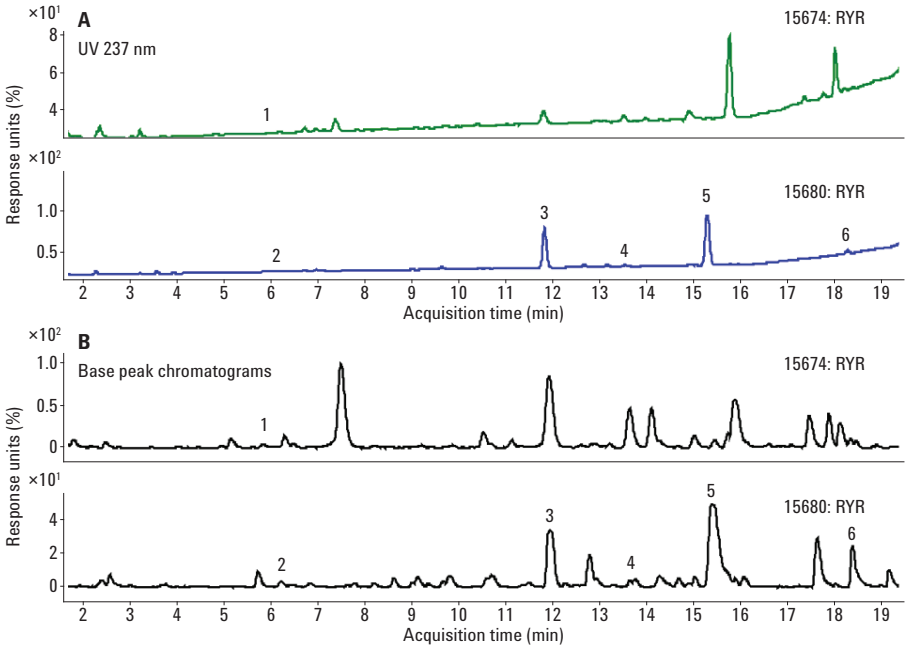

Figure 1. UHPLC DAD (237 nm) chromatogram and Agilent 6530 Q-TOF LC/MS positive ion ESI base peak chromatograms of two RYR samples (CR15674 and AU15680). Citrinin (1), monacolin J (lovastatin diol lactone) (2), monacolin K acid (3), compactin (mevastatin) (4), monacolin K (lovastatin) (5), and dehydromonacolin K (dehydrolovastatin) (6) were found.

With better than 2 ppm mass accuracy obtained, the Agilent 6530 Q-TOF LC/MS system proved valuable in determining compound identities. Molecular formulas were deduced by comparing measured accurate masses to the exact mass values of protonated molecules ($[M+H]^+$). For two isobaric compounds that showed the same nominal mass of *m/z* 405 $[M+H]^+$, the possible formulas of the protonated molecules were $C_{24}H_{36}O_5$ (*m/z* 405.2636) and $C_{23}H_{32}O_6$ (*m/z* 405.2272). Accurate mass values enabled the identification of lovastatin (monacolin K) at *m/z* 405.2636. Confirmation of compound identities were performed based on retention times and MS/MS fragmentation patterns relative to the reference compounds. When standards were not available, compound identities were deduced using accurate mass and accurate mass MS/MS fragments.

Table 1S of the complementary publication available at http://dx.doi.org/10.1016/j.jpba.2014.07.039 provides the detailed qualitative results for the samples for 12 monacolins, 13 pigments, citrinin, and sudan red G [1].

Quantification

Quantification of the three RYR authentic samples by UHPLC DAD revealed that monacolin K and monacolin K acid ranged from 1.9 to 2.3 mg/g and 1.3 to 1.6 mg/g dry weight, respectively. Citrinin and sudan red G were not detected.

The monacolin K and monacolin K acid content of the RYR raw material varied widely from none detected (10 samples) to very high, that is from 0.7 to 24.3 mg/g and 1.3 to 7.6 mg/g (w/w) dry weight, respectively. Citrinin (a mycotoxin) was detected in six of these samples at levels from 10 to 80 µg/g dry weight. Sudan red G was not detected.

All 14 dietary supplements contained monacolin K at levels ranging from 0.03 to 2.62 mg/average capsule or tablet weight, but not citrinin, nor sudan red G. In addition to a large variation in monacolin K found, the dietary supplements contained ratios of monacolins that differed significantly from the authentic samples. The amount of monacolin K expected to be consumed daily through dietary supplement was calculated based on the recommended daily dose on the product label. The total amounts of monacolin K that would be consumed daily based on labeling varied from 0.12 mg/day to 10.46 mg/day.

A complete description of the quantitative results is provided in the complementary publication [1].

PCA

PCA provides exploratory mining and analysis of complex data, enabling discovery and visualization of correlations, differences, and patterns among samples containing numerous variables. Mathematical functions simultaneously reduce data while retaining its discriminating power.

Using MPP software, PCA was performed on the data obtained from the authentic samples and raw materials in the chromatographic region from 5 to 20 minutes. Positive ions with accurate *m/z* values and with a difference corresponding to adducts, isotopes, dimers, or trimers formed in the ion source, or multiply charged species, were merged into MFs as a single variable, called an entity in MPP software (a molecular weight, its retention time, and abundance). Entities were thresholded at 5,000 cps, and those absent in more than 25% of the samples of a given group were removed as a filtering step.

The entities were then filtered on the basis of p-values ($p < 0.05$) calculated by one-way ANOVA. Finally, the remaining entities that satisfied a 4-fold difference in at least one condition pair were selected for PCA. To reduce the relatively large differences in the respective abundant entities, the extracted entities were mean-centered and logarithmically transformed.

Figure 2 shows the scores plot of PCA analysis of the RYR samples by pigment (shown in red) and by monacolin type (shown in blue). The analysis provided a three-dimensional view of three sample groups along with an example of the Q-TOF LC/MS base peak chromatogram of a sample belonging to that group.

Figure 2 shows a large separation between samples along the X-axis. Component 1 explains 84% of the variation found in the Q-TOF LC/MS chromatograms and, thus, the large difference between the groups. Component 2 separated the samples along the Y-axis, and explains 5% of the variation between groups. In the blue group, monacolins were much more prominent than in the red group. As shown in Figure 2, authentic sample AU15680, as did all the authentic samples, fell within the monacolin sample group. In the red group, pigments were more much prominent. Raw material samples CR15674 and CR15676 (in which no monacolin was found) fell in the red sample group. Within that group, a reddish pigment and a yellowish pigment explained the separation of two clusters along the y-axis. It should be noted that while PCA is an unsupervised analysis, the results show that these RYR samples can be modeled by pigment type and monacolin content.

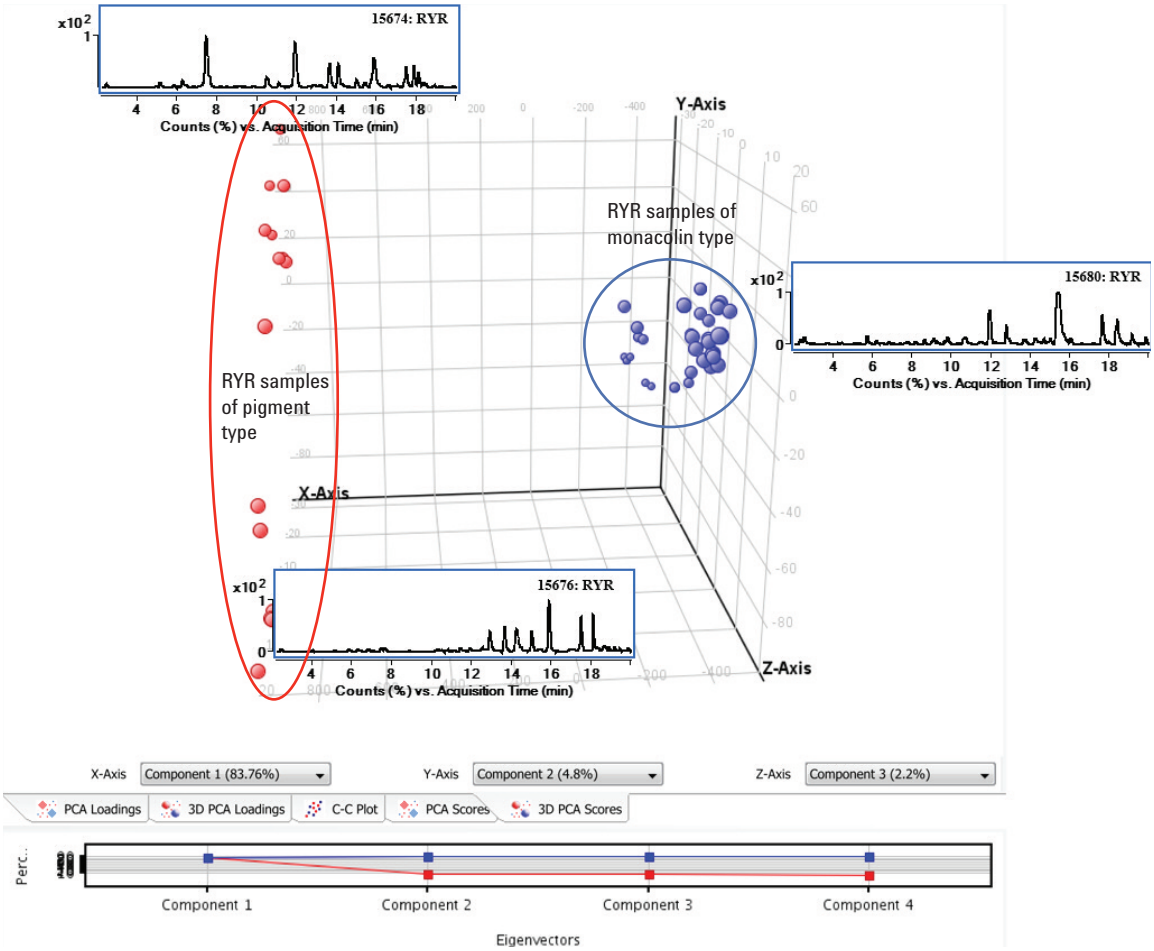

Figure 2. PCA scores plot of RYR authentic and raw material samples by pigment and monacolin type.

Conclusion

Both UHPLC DAD and accurate-mass Q-TOF LC/MS methods uncovered significant differences among RYR samples and raw materials. The dietary supplements analyzed were found to contain variable amounts of monacolins, though the monacolin content was rarely declared on their product labels. While UHPLC DAD is a less expensive technique for quantifying monacolins, sudan dyes, and citrinin, Q-TOF LC/MS uncovered significantly more compounds of interest for analysis and identification. For food safely applications where mycotoxins such as citrinin and adulterants such as sudan red G are monitored at ppb levels, the Q-TOF LC/MS method would be expected to yield lower limits of detection.

High mass accuracy Q-TOF LC/MS data enabled the determination of compound identities, including the identities of isobaric compounds. Identity confirmation was based on retention time and MS/MS fragmentation patterns relative to reference compounds. When standards were not available, compound identity was deduced using accurate mass and accurate mass MS/MS fragments.

Accurate mass Q-TOF LC/MS data processing with MassHunter's automated feature extraction and PCA capabilities could be used to develop a mathematical model to differentiate and classify the RYR samples by monacolin and pigment type. This model could then be used to determine the authenticity and quality of additional RYR samples.

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