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We dedicate this paper to our colleague, Prof. Sandra Apers, who passed away much too early on February 5th, 2017.

Abstract

A robust analytical method, using reversed phase high-performance liquid chromatography with diode array detection, was developed and validated for the quantification of monacolins in red yeast rice bulk products. Tests on the composition of the extraction solvent, extraction time and the number of repetitions of extraction were evaluated with the aim of complete extraction of the monacolins and minimal transitions between the monacolins during analysis. Monacolin K (acid form), monacolin K (lactone form) and minor monacolin peaks were separated on a C18 column (250 x 4.6 mm, 5 µm) using acetonitrile / 0.1% trifluoroacetic acid

as the mobile phase. For the calibration curve of monacolin K (lactone form), a linear correlation in the range 6-119 µg/mL was found. The precision of the method for time and concentration gave a relative standard deviation of less than 5%, which was deemed acceptable. The recovery of the method was 98.75%.

Keywords: Red yeast rice, monacolin K, HPLC, validation

Chemical compounds studied in this article: Monacolin K (PubChem CID: 53232)

1. Introduction

Red yeast rice (RYR) is produced by fermentation of rice with *Monascus* species. During fermentation, a variety of secondary metabolites are formed: pigments, citrinin, monacolins and others. In Asia, RYR has been used in food as well as a medicine for thousands of years. Recently, RYR products have been described in scientific reports for the management of blood cholesterol, diabetes, blood pressure, obesity, Alzheimer's disease and the prevention of cancer development (Shi & Pan, 2011; Kalaivani, Sabitha, Kalaiselvan, & Rajasekaran, 2010; Verhoeven, Lopez Hartmann, Remmen, Wens, Apers, & van Royen, 2013; Verhoeven et al., 2015). In 2011, EFSA concluded that a cause-and-effect relationship had been established after consumption of monacolin K from RYR, and the maintenance of normal blood LDL cholesterol concentrations. This effect can be achieved by daily consumption of 10 mg of monacolins, i.e. sum of acid and lactone form (Figure 1) from fermented RYR (EFSA Journal 2011). Because of its effect on hypercholesterolemia, RYR is gaining popularity in Western society and entering the market as a food supplement.

For companies providing food supplements containing RYR, it is very important to control the quality of the RYR bulk product, which is produced mostly in China. Several analytical

methods for the quantification of monacolins (acid form and lactone form) in RYR have already been described in literature, using high-performance liquid chromatography (HPLC) with ultraviolet (UV), photodiode array (PDA) or mass spectrometric (MS) detection (Li, Zhang, Wang, & Hu, 2004; Li, Liu, & Wang, 2005; Ou, Wang, & Lai, 2009; Huang, Hua, Boa, & Xie, 2006; Huang, Xu, Li, & Wang, 2010; Wu, Kuo, Lee, Hsu, & Pan, 2011). In applying these methods, we experienced problems with their robustness for the analysis of RYR bulk products. Causes we identified were 1) incompleteness of extraction and 2) pH-dependent transitions, i.e. we could find a transition of monacolin K (lactone form) into its acid form if the samples were prepared in a solution with an uncontrolled pH. Therefore, it was necessary to develop and validate a more robust method for the determination of monacolin K (lactone and acid form) in RYR bulk products. Additionally, we found that RYR bulk products should be kept in the refrigerator prior to analysis since our results indicated a decrease in monacolin K content if RYR bulk products were kept at room temperature.

2. **Material and methods**

2.1.Chemicals, Reagents, materials

Acetonitrile (ACN) HPLC grade, absolute Ethanol (EtOH), Methanol (MeOH) HPLC grade and Trifluoroacetic acid (TFA) were purchased from Acros Organics (New Jersey, USA) and MilliQ water (MilliQ) was prepared with an in-house MILLI-Q Reference A+ water purification system (Millipore, Bedford, MA, USA). The monacolin K (lactone) standard and lovastatin for peak identification CRS standard were obtained from EDQM Council of Europe (Strasbourg, France). RYR bulk product was kindly provided by Phacobel (Soheit-Tinlot, Belgium) and Omega Pharma (Nazareth, Belgium), and stored at 4°C prior to analysis. A buffer solution pH 7 was prepared with 58.2 mL NaOH (0.1M) (VWR International, Radnor, USA)

and 100 mL KH_2PO_4 (0.1M) (VWR International, Radnor, USA). The extraction solvent was prepared with ACN and the buffer solution pH=7 (6:4).

2.2.Equipment

An Agilent 1260 HPLC system of Agilent Technologies (Santa Clara, USA) with a diode array detector (detection at 237 nm) was used for the analysis, with a reversed phase (RP) C18 column (GraceSmart™, Grace Davison (Alltech), (250 x 4.6 mm, 5µm) at a temperature of 20°C and a flow rate of 1.0 mL/min. Solvent A (0.1% TFA) and solvent B (ACN) were used with the following gradient: in 20 min from 40% B to 75% B, 10 min 75% B, from 75 to 100% B in 5 min, 3 min at 100% B, from 100 to 40% B in 2 min and 5 min 40% B.

2.3.Sample preparation

Samples (150 mg) were extracted by ultra-sonication with 15 mL of solvent for 30 minutes. After centrifugation, the upper layer was decanted. This procedure was repeated twice; the fractions were combined and made up to 50.0 mL with the extraction solvent. 20 µL was injected on the HPLC for analysis. Samples were kept in the dark and at -20°C until HPLC analysis.

2.4.Preparation of standard

5.0 mg monacolin K (lactone) CRS was dissolved in 10.0 mL of extraction solvent. 1.0 mL of the resulting solution was diluted to 20.0 mL with the extraction solvent.

2.5.Method validation

For the method validation, ICH guidelines on the validation of analytical methods (ICH guidelines 1994, 1996) were followed. The results are given as percentages in Table 1, with n

- the number of values. Excel 2013 (Microsoft Office) was used for statistics and a 5% level of significance was selected.

2.5.1. Calibration model

The standard monacolin K (lactone) was prepared at 6 concentrations, from 6.0 to 119.2 µg/mL. These samples were analyzed in duplicate.

2.5.2. *Precision*

Six independently prepared samples of 100% (150 mg) were analyzed on three different days according to the method described in 2.3 to determine the repeatability and the inter-day precision. Six samples weighing half of the mass (75 mg) and six samples weighing 150% (225 mg) were analyzed according to the newly developed method to evaluate precision at different concentrations. Two different batches with different ratios of monacolin K (acid and lactone form), specifically 0.486% monacolin K (acid) and 2.80% monacolin K (lactone) (Batch A) and containing 1.01% monacolin K (acid) and 1.88% monacolin K (lactone) (Batch B), were analyzed.

2.5.3. Accuracy

A recovery experiment was done to evaluate the accuracy of the method: monacolin K (lactone) was added at 50, 100 and 125% to 50% of the RYR bulk product. Three independently prepared samples at each concentration were analyzed using the method developed.

3. **Results and Discussion**

For the quality control of RYR bulk powder, the content of monacolins and the ratio lactone/acid form are important. Thus, the aim of this work was to develop a robust method that achieved complete extraction and prevented transition of these compounds during analysis.

3.1.Method development

The extraction of monacolin K (acid) and monacolin K (lactone) from RYR bulk powder with EtOH:H_2O, ACN or MeOH has been described elsewhere (Li et al., 2005; Ou et al., 2009; Wu et al., 2011, Heber, Lambertas, Lu, Bowerman, & Liang, 2001 and Zheng, Xin, & Guo, 2009). To develop a robust quantitative method, we tested the ratio sample:solvent, time (30, 60 and 90 minutes), composition of the extraction solvent, and number of extractions needed. The extraction solvent resulting in the highest monacolin content was EtOH:H_2O 75:25, confirming earlier research (Li et al., 2005). The extraction efficacy, though, could be increased by reducing the amount of sample and increasing the solvent volume (150 mg in 15 mL instead of 500 mg in 8 mL; Li et al., 2005). Repeating the extraction twice guaranteed completeness of extraction and repeatable results. Increasing the extraction time to 60 and 90 minutes did not improve the extraction. If samples were measured after several hours, we could see the conversion of the lactone into the acid when using EtOH:H_20 (75:25) as the solvent. Therefore, to increase recovery of monacolins in routine analysis for a higher number of samples, different extraction solvents were tested. Based on the fact that the lactone is converted into the acid under acidic conditions (Huang at al., 2010), we tested the influence of pH on the stability of the samples. We found that the profile of monacolins remained stable for at least 8 hours at room temperature when H_2O in the extraction solvent was replaced with a buffered solution (pH=7). Since we observed instability, i.e. phase separation after a few hours, in the extraction solvent consisting of EtOH:buffer (pH=7) (75:25), we switched to acetonitrile. The extraction solvent ACN:buffer (pH=7) in a ratio 6:4 resulted in yields of monacolins (the lactone and acid form)

equal to EtOH:H_2O (75:25). Without the presence of the buffer, the lactone was easily converted to the acid.

The HPLC gradient was optimized on a GraceSmart™ column of 250 x 4.6 mm (5µm particles). To remove more apolar compounds that might be present in bulk products or finished products of RYR, we adapted slightly the gradient applied by Li et al. (2005): acetonitrile at the end of the run was increased from 75% to 100%. This resulted in less carry-over. Figure 2A shows a chromatogram of the lovastatin for peak identification CRS standard. The peaks for monacolin K (lactone) and impurities a, b and f are indicated, where impurity b is the acid form of monacolin K. Additionally, the acid and lactone form are indicated in the chromatograms for RYR from batch A (Fig. 2B) and batch B (Fig. 2C).

During the validation of the method, more specifically when investigating the intermediate precision with respect to time and concentration, we found unrepeatable results that could be pinpointed to a decrease in monacolin K (acid form) in the bulk sample. Indeed, high temperatures and high humidity had already been described to have an influence on the stability of monacolin K in the production of RYR bulk products: monacolins decreased significantly under conditions of high humidity, high temperatures and sunlight (Li et al., 2005). Therefore, it is important to keep RYR bulk products refrigerated.

To summarize, in the final method, monacolin K (acid form) and monacolin K (lactone form) were extracted from the bulk RYR powder by sonication of 150 mg bulk material in the extraction solvent (15 mL) for 30 minutes. After centrifugation, the upper layer was decanted in a volumetric flask of 50.0 mL. The extraction procedure was repeated twice, using the same volume of fresh solvent. The extracts were combined and made up to 50.0 mL with the solvent before 20 µL of the final solution was injected on to the HPLC- system.

3.2.Method validation study

For the validation of the method on the RYR bulk powder, ICH guidelines on the validation of analytical methods (ICH guidelines 1994, 1996) were followed.

3.2.1. Calibration model – response function

Monacolin K (lactone form) reference solutions at 6 concentrations (6.0 to 119.2 µg/mL) were analyzed in duplicate to examine linearity. The least square line and correlation coefficient were calculated using the linear regression function. Based on graphical inspection of the calibration curve (area versus concentration, Fig. 3), and since the residuals (Fig. 4) fulfilled the requirement of homoscedasticity and were randomly scattered, a linear model could be applied. A single-point calibration was justified since point (0) fell within the calibration curve.

3.2.2. Precision

The repeatability (i.e. the precision under the same conditions over a short interval of time) and the intermediate precision (i.e. the effects of performing analysis on different days) were investigated for both the monacolin K acid and lactone form.

For each day, the standard deviation and relative standard deviation (RSD%) were calculated. Within and between day RSD%s are summarized in Table 1. These results showed good repeatability for determination of the total content as well as contents of the acid and lactone form separately. The Cochran's values calculated showed that variances of the results on the three different days for both batches were equal and ANOVA could be applied. ANOVA showed a significant difference between the results obtained on the three different days. Although the RSD%s (within and between) were higher than the limit set by Horwitz (Albert & Horwitz, 1997), these were less than 3% for all determinations, except for monacolin K (acid form) in batch A. However, since the RSD%s were less than 5%, it was still acceptable for the analysis of a complex plant matrix and the method can be considered precise.

Six samples weighing half the mass (50%) and six weighing 150% were analyzed to evaluate the precision of the method over the whole range. At each level, the standard deviation and RSD%s were calculated. Cochran's test showed that the variation was equal over the whole range of the method. An ANOVA single factor showed the results were significantly different. Within and between level RSD%s were calculated and are shown in Table 1. The RSD% between levels was in the same order as the RSD% between days and, therefore, acceptable. Linearity of the method across this broad range confirmed the completeness and robustness of the extraction of monacolins from the RYR matrix.

3.2.3 Accuracy

Accuracy of an analytical method expresses the closeness of agreement between values that are accepted as conventional true or an accepted reference value and the value found (ICH guidelines 1994, 1996).

A recovery experiment was performed, adding three different concentrations of the monacolin K (lactone form) to the samples at the start of the extraction. A mean recovery % (n=9) of 98.75% (RSD% = 1.56%) was obtained and no dependency on the concentration was seen. Although 100% was statistically not included in the confidence interval, the method can still be considered accurate since it was within the generally accepted range 97-103%.

Conclusions

An HPLC method to analyze monacolin K (the lactone and acid form) in red yeast rice bulk powder was optimized. The extraction procedure was optimized to guarantee completeness of extraction and stability of the compounds during analysis. The method was validated according to ICH guidelines. The method was linear in the range 6-119 µg/mL and had a recovery of

98.75%. Repeatability and accuracy were acceptable. This method can be used to control the quality of RYR bulk products and would allow companies marketing products containing RYR to use an accurate and reliable method of analysis that meets ICH standards. Based on this method, companies could develop and validate a method for quality control on their commercial products. In analyzing RYR bulk materials used in supplements and available on the Belgian market, the total concentration of monacolin K (lactone and acid form) ranged from 0.33 – 3.61%. The products had large differences in chromatographic profile, i.e. from the pure monacolin K lactone form to a profile showing other monacolin peaks next to the two expected, and a significant difference in ratios between the monacolin K acid and lactone form. The question to be investigated further is the best profile and ratio for use of these preparations to maintain and support a healthy cholesterol level.

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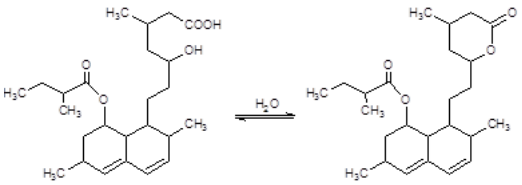

Monacolin K
Acid form

Monacolin K
Lactone form

Figure 1: The acid and lactone form of Monacolin K.

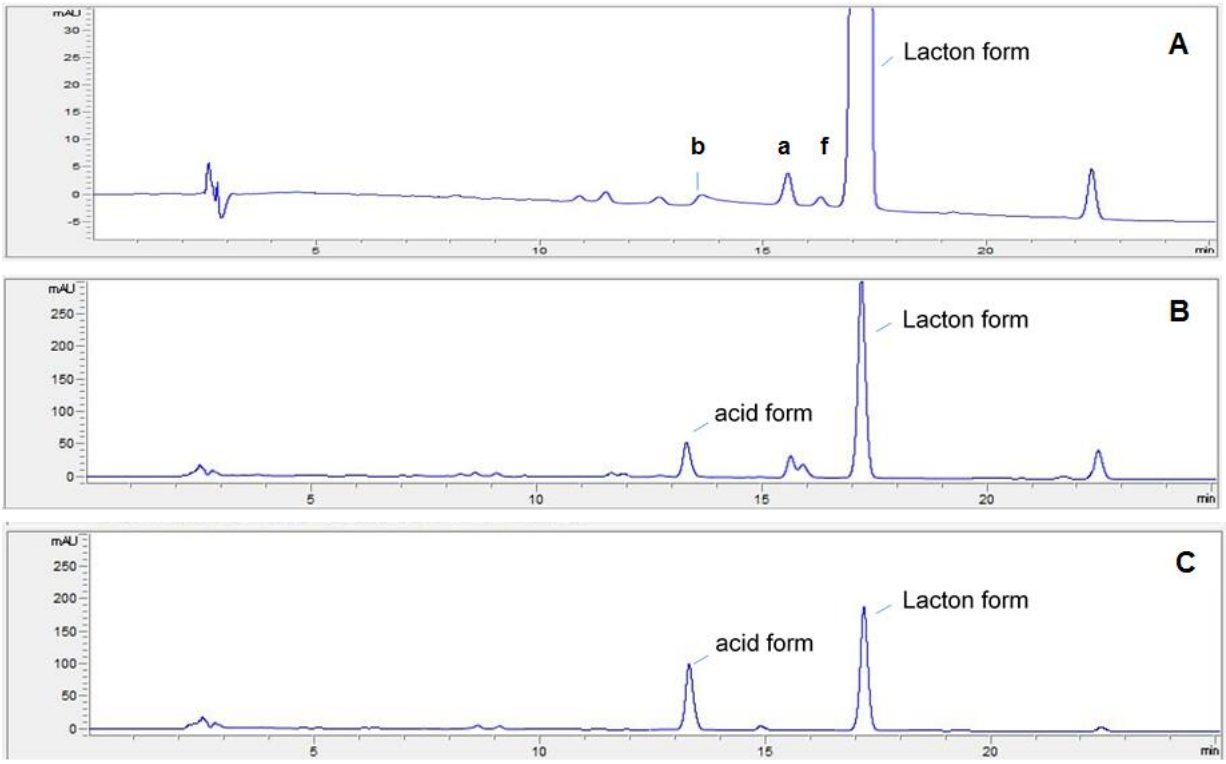

Figure 2: **A**/ Chromatogram of Lovastatin for peak identification CRS1 – LC for related substances, impurities a, b and f are indicated; **B**/ Chromatogram of Batch A with the lacton and acid form of monacolin K indicated; **C**/ Chromatogram of Batch B with the lacton and acid form of monacolin K indicated.

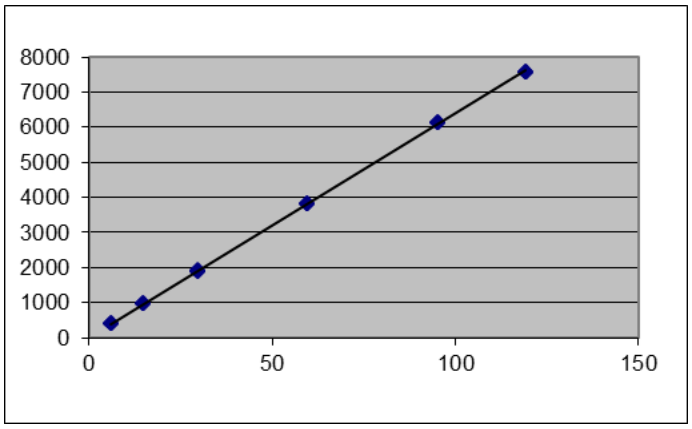

Figure 3: Least square line for the 6 concentrations (6.0, 14.9, 29.8, 59.6, 95.4 and 119.2 µg/mL), with y = 63.705 x + 11.635

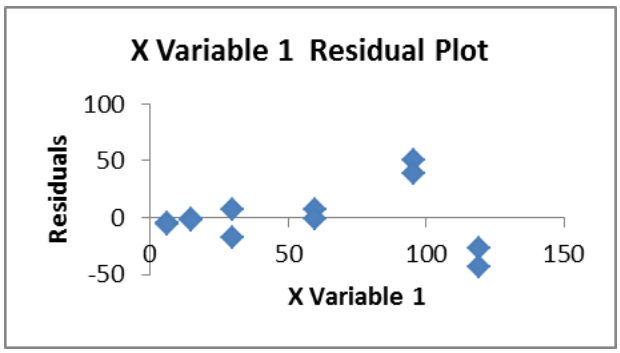

Figure 4: Residual plot – residuals are randomly scattered and homoscedasticity is fulfilled.

Table 1: Validation results of the precision on different days and different concentration levels.

	Batch A							Batch B						
Parameter	Lactone			Acid form			Total	Lactone			Acid form			Total
Mean content (%)	2.80			0.49			3.29	1.88			1.01			2.89
Precision on different days (n = 3)														
Repeatability														
Number of replicates	6			6				6			6			
RSD% (day1/day2/day3)	0,41	0,75	0,52	0,96	1,02	0,96		0,48	0.32	0.30	0.52	0.62	0,48	
Intermediate precision														
Number of days	3			3			3	3			3			3
Number of replicates	6			6			6	6			6			6
RSD% $_{\text{between groups}}$	2.76			4.22			2.74	2.50			2.23			2.19
RSD% $_{\text{max}}$ (2/3 Horwitz)	2.28			2.97			2.23	2.43			2.66			2.27
F_{calc} (F_{crit} = 3.68)	132			105			136	265			95			221
Precision on concentration levels														
Repeatability														
Number of replicates	6			6				6			6			
RSD% (50%/150%)	0,52	0,80		1,05	1,78			1.68	0.51		0.98	0.55		
Intermediate precision														
Number of days	5			5			5	5			5			5
Number of replicates	6			6			6	6			6			6
RSD% $_{\text{between groups}}$	2.30			4.17			2.34	1.95			1.70			1.66
RSD% $_{\text{max}}$ (2/3 Horwitz)	2.28			2.97			2.23	2.43			2.66			2.27
F_{calc} (F_{crit} = 2.76)	77			66			76	27			35			52