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Stable isotope ratio analysis for authentication of red yeast rice

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ABSTRACT

Red yeast rice (RYR) is a dietary supplement obtained from rice fermented with the mould *Monascus purpureus*. It contains Monacolin K which is a hypocholesterolemic statin used to prevent cardiovascular diseases. The homologous prescription biosynthetic statin, lovastatin, is not chemically distinguishable from monacolin K.

In this work we investigated whether $\delta^{13}C$ and $\delta^{2}H$ can distinguish monacolin K from lovastatin and can detect the presence of lovastatin in RYR.

18 samples of red yeast rice powder and 18 samples of lovastatin were collected. Monacolin K was isolated from RYR using preparative HPLC and together with lovastatin, was subjected to analysis of $\delta^{13}C$ and $\delta^{2}H$ using Isotope Ratio Mass Spectrometry.

Thanks to the different photosynthetic cycles of the matrices used for their synthesis, monacolin K and lovastatin have different $\delta^{13}C$ values (−29.6‰ ± 0.6 and −16.7‰ ± 2.6 respectively). $\delta^{2}H$ is significantly ($p < 0.001$) lower in monacolin K but the ranges of values partially overlap with those of lovastatin. By defining a $\delta^{13}C$ threshold value of −28.3‰ for monacolin K, addition of lovastatin from a minimum of 10% can be identified.

$\delta^{13}C$ analysis can be therefore proposed as a suitable tool for detecting the authenticity of RYR on the market.

1. Introduction

Red yeast rice (RYR) is a fermented food product produced by inoculating *Monascus purpureus* mould into steamed rice of different varieties [1]. It has been used for centuries as a food as well as a traditional Chinese medicine to invigorate the body, aid digestion, and revitalize the blood [2]. Extracts of RYR are well known for their content of starch, sterols, isoflavones, monounsaturated fatty acids and monacolins [3]. It contains fourteen naturally occurring monacolins: J, K, L, M, and X, as well as their hydroxy acid forms and dihydromonacolin K, L, compactin, 3alpha-hydroxy-3,5-dihydromonacolin L [4]., They have a similar structure: [8-[2-(4-hydroxy-6-oxo-oxan-2-yl) ethyl] −3,7-dimethyl- 1,2,3,7,8,8 of a-hexahydronaphthalen-1-yl] with different types of substituent in position 1 (e.g. −OH for monacolin J or −H for monacolin L). Of these polyketide derived compounds, monacolin K and its dihydro derivative are the most pharmacologically active [5].

Monacolin K (Fig. 1) is a potent competitive inhibitor of Hydroxymethylglutaryl-CoA Reductase (HMG-CoA reductase) [6], that catalyzes the reduction of HMG-CoA to mevalonate during the synthesis of cholesterol [7], and has been demonstrated to be effective in reducing both cholesterol and triglycerides. For this reason it is effective in the treatment of cardio-cerebrovascular diseases caused by hyperlipidemia and atherosclerosis [8].

In the 1970s, the Japanese microbiologist Akira Endo and researchers at the pharmaceutical company Merck & Co. in the United States isolated monacolins from *Monascus purpureus,* the same fungus used to make RYR but cultured under carefully controlled conditions, and the first commercially marketed statin, lovastatin (mevinolin, MK803), from *Aspergillus terreus* [9,10]. Lovastatin became the patented, prescription drug Mevacor for Merck & Co., whereas RYR remained a non-prescription dietary supplement in the United States and other countries.

The use of RYR has become very common as an alternative to statin therapies with lovastatin. The increase in use may be attributed to a number of factors, including the population's perception of RYR as a "natural" therapy compared to lovastatin.

For this reason, while to date the European Commission has not

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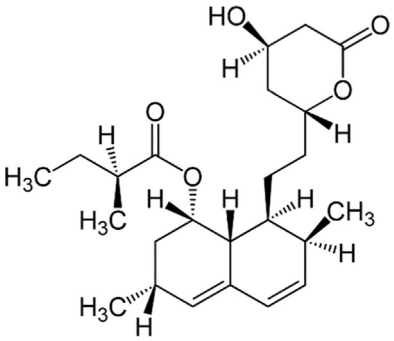

Fig. 1. Monakolin K structure.

legislated on limits for these supplements and there is no standardisation in European countries, the Food and Drug Administration (FDA) has stated since 2007 [11] that RYR products containing monacolin K are identical to a drug and therefore subject to regulation. As regards the therapeutic efficacy, a 2011 EFSA opinion confirmed that a 10 mg daily intake of monacolin K was beneficial in maintaining normal cholesterol levels [12].

In any case, reaching this pharmacologically effective level in RYR is not easy, due to a lack of standardized production methods for this product, which is still produced using traditional solid-state fermentation (SSF) of the cooked whole rice kernel [13,14]. The concentration of monacolin K depends on both the length and conditions of the fermentation process and it often varies from one lot to another [13]. Gordon et al. and Avula et al. [15,16] confirmed this problem and showed how RYR products on the market have variable contents of monacolin K, which does not always reach the pharmacological level.

In this context, there is a suspicion that RYR products could be spiked with purified lovastatin, identical to monacolin K, without declaring thid, to obtain a product with the pharmacologically effective monacolin K content.

To date, only one method has been proposed to detect this spiking. Different authors [4,16], have reported that Chromatographic Chemical Profiling is an effective and convenient approach for qualitative evaluation of RYR. This is based on the fact that in RYR many monacolin compounds (e.g. J, L, M, and X) beside K, should be found. When only Monacolin K is detected, as in the case of several commercial products [4,16], the suspicion of non-authenticity arises. The limit of this approach is that it is not possible to determine if the statin is natural monacolin K or the biosynthetic lovastatin.

For the first time, in this study we investigated the possibility of distinguishing monacolin K from lovastatin using stable isotope ratio analysis. This analysis has found widespread application in food science in recent years [17], in particular to differentiate between natural and synthetic or biosynthetic ingredients [18–22].

18 samples of authentic red yeast rice powder and 18 samples of biosynthetic lovastatin were collected, taking into consideration the main producers of RYR and lovastatin. Monacolin K was isolated from rice using preparative HPLC and, together with lovastatin, subjected to analysis of the isotopic ratios of C and H using an Isotope Ratio Mass Spectrometer interfaced with an Elemental Analyser and a Pyroliser. The limited availability of authentic samples, especially of RYR, from international producers did not make it possible to build up a more extensive database.

2. Material and methods

2.1. Sampling

15 commercial pure biosynthetic lovastatins with purity of > 95%, 3 drug containing biosynthetic lovastatin and 18 RYR powders were collected. The samples of lovastatin were bought directly from pharmaceutical companies or their certified vendors, taking into consideration at least one sample for each trademark on the market. The samples of authentic RYR powder were provided by different suppliers, which guaranteed their authenticity: 7 samples were provided by the Italian importer ALESCO Srl, Pisa, Italy and the others came directly from the five main Chinese producers.

The samples of pure lovastatin were analysed directly without extraction, whereas both the monacolin K and lovastatin of drugs were extracted according to the following method.

2.2. Extraction of monacolin K

The method was adopted by Li et al. [4]. About 1.5 g of red yeast rice powder or drug tablet pulverized with a mill was transferred to a 50-ml glass beaker and extracted with 50 ml of 75% ethanol for 30 min in an ultrasonic bath and subsequently centrifuged for 10 min at 3000 rpm. This extraction procedure was repeated twice, and the total supernatant was transferred to a 200 ml volumetric flask. The final solution was decanted for 30 min, filtered through a 0.45-μm membrane and then blown to dryness with rotary vapour. The residue was recovered with methanol and the lovastatin was extracted with preparative HPLC.

2.3. HPLC Separation

Preparative HPLC separation was performed by injecting 2 ml of filtered extract dissolved in methanol onto A Supelco Discovery (21 × 250 mm, 5 μm) HS C-18 reversed-phase HPLC column (Supelco, Bellefonte, PA). Elution was performed using Interchim Kromaton Puriflash model 4250 pumps with gradient elution consisting of distilled water (eluent A) and acetonitrile (eluent B) at a flow rate of 10 l per minute. The gradient pattern consisted of a linear gradient from 35% to 75% B in 20 minutes, keeping 75% B from 20 minutes to 30 minutes. The percentage of acetonitrile was then raised to 100% over 5 minutes and run isocratically for 15 minutes to purge the column. The total analysis time was 60 minutes including column stabilization. Monacolin K was detected by absorbance at 237 nm and was collected between 30 and 33 min.

2.4. Stable isotope analysis

The $^{13}C/^{12}C$ ratio was measured (around 0.5 mg) using an isotope ratio mass spectrometer (IsoPrime, Isoprime Limited, Germany) following total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited, Germany). The $^{2}H/^{1}H$ ratio was measured (around 0.3 mg) using an IRMS (Finnigan DELTA XP, Thermo Scientific) coupled with a Pyrolyser (Finnigan TC/EA, high temperature conversion elemental analyser, Thermo Scientific), following the method described elsewhere for olive oil [23].

The values are denoted in delta in relation to the international V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$, V-SMOW2 (Vienna-Standard Mean Ocean Water number 2) and SLAP2 (Standard light Antarctic Precipitation number 2) for $\delta^{2}H$, according to the following general equation:

$$\delta i\ \mathrm{E} = \frac{(\mathrm{i\ RSA} - i\ \mathrm{RREF})}{\mathrm{i\ RREF}}$$

where i is the mass number of the heavier isotope of element E (^{13}C and ^{2}H);

RSA is the respective isotope ratio (for C: number of ^{13}C atoms/number of ^{12}C atoms or as $^{13}C/^{12}C$ approximation and for H: number of ^{2}H atoms/number of ^{1}H atoms or as $^{2}H/^{1}H$ approximation) of a sample and RREF is the relevant internationally recognised reference material [24]. The delta values are multiplied by 1000 and expressed in units “per mil” (‰).

Sample analysis was carried out in duplicate. For $\delta^{13}C$ the isotopic