International Food Research Journal 28(6): 1276 - 1284 (December 2021)
Journal homepage: *http://www.ifrj.upm.edu.my*

An improved method for the purification and structural analysis of rubropunctatin from red yeast glutinous rice

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Article history

Received: 1 March 2020
Received in revised form: 27 January 2021
Accepted: 24 May 2021

Keywords

purification, structural analysis, salmon pink pigments, rubropunctatin, red yeast glutinous rice

Abstract

An improved method for the separation and purification of rubropunctatin from red yeast glutinous rice was developed. In this method, silica gel column chromatography and thin layer chromatography were used to separate the *Monascus* pigments. Pure methanol and a mixture of ethyl acetate and methanol (13:7, v/v) were selected as the eluent and running solvent, respectively. When compared with previously reported methods, the developed method required fewer types of solvents, and could be suitable for large-scale laboratory production. The extracted pigment was analysed by ultra-high performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-TOF-MS) and nuclear magnetic resonance (NMR) (^{1}H NMR, ^{13}C NMR). Structural analysis revealed a molecular mass of 353.2 *m/z* $[M-H]^-$, and a structure including an azaphilone body with two side chains. This structure was consistent with that reported for rubropunctatin. Therefore, the improved purification method reported herein could efficiently be used for the extraction of rubropunctatin from red yeast glutinous rice.

Introduction

Colours occupy major position in numerous industries such as food, cosmetic, medicine, and textile (Venil *et al.,* 2014). In the past few decades, synthetic pigments have been widely used. Contrary to natural pigments, synthetic pigments have been associated with safety problems in the food industry (Tuli *et al.,* 2015). Many synthetic pigments have been banned worldwide due to adverse effects such as hypersensitivity, carcinogenicity, and other toxicological effects. Hence, consumers have a growing demand for the use of natural pigments. Anthocyanins are common in our daily diet especially in cereals, vegetables, and fruits of all colours (Wu *et al.,* 2017). Anthocyanins are classified in the flavonoid group of polyphenols. Recently, anthocyanins have aroused scientific interest due to their health-promoting properties in humans (Li *et al.,* 2017). Anthocyanins act as antioxidants, enhance cell activity, and extend life (Li *et al.,* 2019; Anh *et al.,* 2019). However, anthocyanins are plant-derived pigments, and their production is affected by the season, yield, manpower consumption, as well as complex extraction processes (Arulselvi *et al.,* 2014). In this context, microorganism-derived pigments could be interesting alternatives. *Monascus* pigments are secondary metabolites of the moulds *Monascus* spp. (Patakova, 2013). *Monascus* pigments display anticancer and antimicrobial properties as well as many other biological activities (Lee and Pan, 2012; Patakova, 2013; Vendrusculo *et al.,* 2014; Chen and Wu, 2016). Nowadays, *Monascus* pigments are mostly extracted from *Monascus* strains (Jůzlová *et al.,* 1996; Hajjaj *et al.,* 2000; Blanc *et al.,* 2010) and red yeast rice which is produced by growing *Monascus* spp. on rice (Huang *et al.,* 2016). To date, many *Monascus* pigments remain to be identified which hinders further research on their functionality and stability. Therefore, it is imperative to decipher *Monascus* pigments' composition by implementing efficient separation and purification techniques.

The six classical pigments of *Monascus* are monascin and ankaflavin (yellow), monascorubrin and rubropunctatin (orange), and monascorubramine and rubropunctamine (red) (Feng *et al.,* 2012). Little attention has been paid to the separation and purification of the rubropunctatin's monomer, although many research works have focused on the purification of novel *Monascus* pigments. Lian *et al.*

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DOI: https://doi.org/10.47836/ifrj.28.6.19

(2007) prepared crude extracts of *Monascus* using filter membranes (80 hole/cm^2) and lyophilisation. Then, new red pigments were directly extracted with methanol, ethyl acetate, and hexane from the crude extracts. After that, two new *Monascus* red pigments were separated by two-dimensional thin layer chromatography (TLC) with petroleum ether:ethanol (7:3, v/v) and dichloromethane:methanol (1:1, v/v) as the eluents (Lian *et al.,* 2015). Huang *et al.* (2014) extracted two novel orange pigments with yellow fluorescence by column chromatography and TLC. The silica gel used for column chromatography was 200 - 300 mesh. Hexane and ethyl acetate (7:3, v/v) were used for the elution, while *n*-hexane and ethyl acetate (7:3, v/v) were used for TLC (silica gel 60 plates). Yang *et al.* (2018) obtained water-soluble *Monascus* yellow pigments by TLC. However, the pigments obtained eventually were all a mixture of two or more pigments according to other reports (Lian *et al.,* 2007; Huang *et al.,* 2014; Yang *et al.,* 2018), and none of them could be completely separated into their monomeric form. Furthermore, the reagents used in these methods were relatively numerous and toxic.

The aim of the present work was therefore to simplify the preparation of the eluent and running solvent for silica gel column chromatography and TLC to reduce the reagents used and their harmfulness. Meanwhile, a novel method was explored to separate *Monascus* pigments from red yeast glutinous rice to obtain a pigment monomer. The structure of the separated pigment obtained in the present work was consistent with that of rubropunctatin (Feng *et al.,* 2012). Therefore, the developed method could be suitable for obtaining the rubropunctatin monomers. The present work also laid a foundation for the study of the functions and other characteristics of rubropunctatin. At the same time, it also provided a certain knowledge base for the applications of rubropunctatin in the production of beverages, meat products, and in other industries.

Materials and methods

Materials and reagents

The long-grain glutinous rice used in the present work was obtained from Bengbu Brother Grain and Oil Company, China. Acetonitrile and methanol (HPLC grade) were supplied by Tedia, USA. Distilled water was produced by the Guangzhou Watsons Food and Beverage Company. Methanol-d4 was obtained from ICON Isotopes (Summit, NJ). All the other reagents and solvents were of analytical grade, and purchased from Shanghai Titan Scientific Company.

Preparation of red yeast glutinous rice

The *Monascus* strain ACCC 30352 was supplied by Shandong Yiyuan Kangyuan Biotechnology Company, and its cultivation was carried out following the methods of Wu *et al.* (2011) with slight modifications. Briefly, the mould was maintained and cultivated on solid medium (49% glucose, 16% peptone, 25% soluble starch, 10% agar) at 25°C for 7 d. Then, its spores were harvested with sterile water, seeded into 150 mL of liquid medium (90% sterile water, 5% glucose, 2% peptone, 3% soluble starch), and cultivated with shaking (150 rpm) at 25°C for 4 d.

Next, the long-grain glutinous rice was soaked in acetum (pH 3.7) for 12 h, and 30 g were drained and packed into a 100-mL Erlenmeyer flask, and sterilised according to the standard procedure (121°C, 30 min). After cooling, 3 mL of the prepared seed cultures were transferred into the flasks. Samples were fermented at 30°C to produce red yeast glutinous rice. After 7-d incubation, samples were harvested, dried at 45°C, and ground into powder for further extraction.

Extraction and separation of Monascus pigments

Two grams of red yeast glutinous rice powder were soaked into 400 mL of distilled water, and placed in a beating machine for 5 min. The mixture was then poured into a 500-mL beaker, and placed into a CNC ultrasonic cleaner (KQ-250DE, Kunshan Ultrasonic Instrument Company, China) at room temperature for 30 min. The procedure was repeated twice. The large-grained red yeast glutinous rice powder was separated from the mixture by filtration (100 hole/cm^2). The solutions were subsequently centrifuged at 25°C for 30 min at 10,000 rpm. The supernatants were finally evaporated under vacuum.

Mukherjee and Singh (2011) obtained a new red pigment from *M. purpureus* by silica gel column chromatography and TLC. The present work repeated these separation and purification processes, and the workflow is shown in Figure 1A (pigments A, B, and C did not have specific names; they were three different mixtures of pigments isolated from the extracted crude pigment. Mukherjee and Singh (2011) did not name them either). This purification process was complex.

The isolation and purification techniques developed in the present work were also adapted from Mukherjee and Singh (2011), and the workflow is shown in Figure 1B.

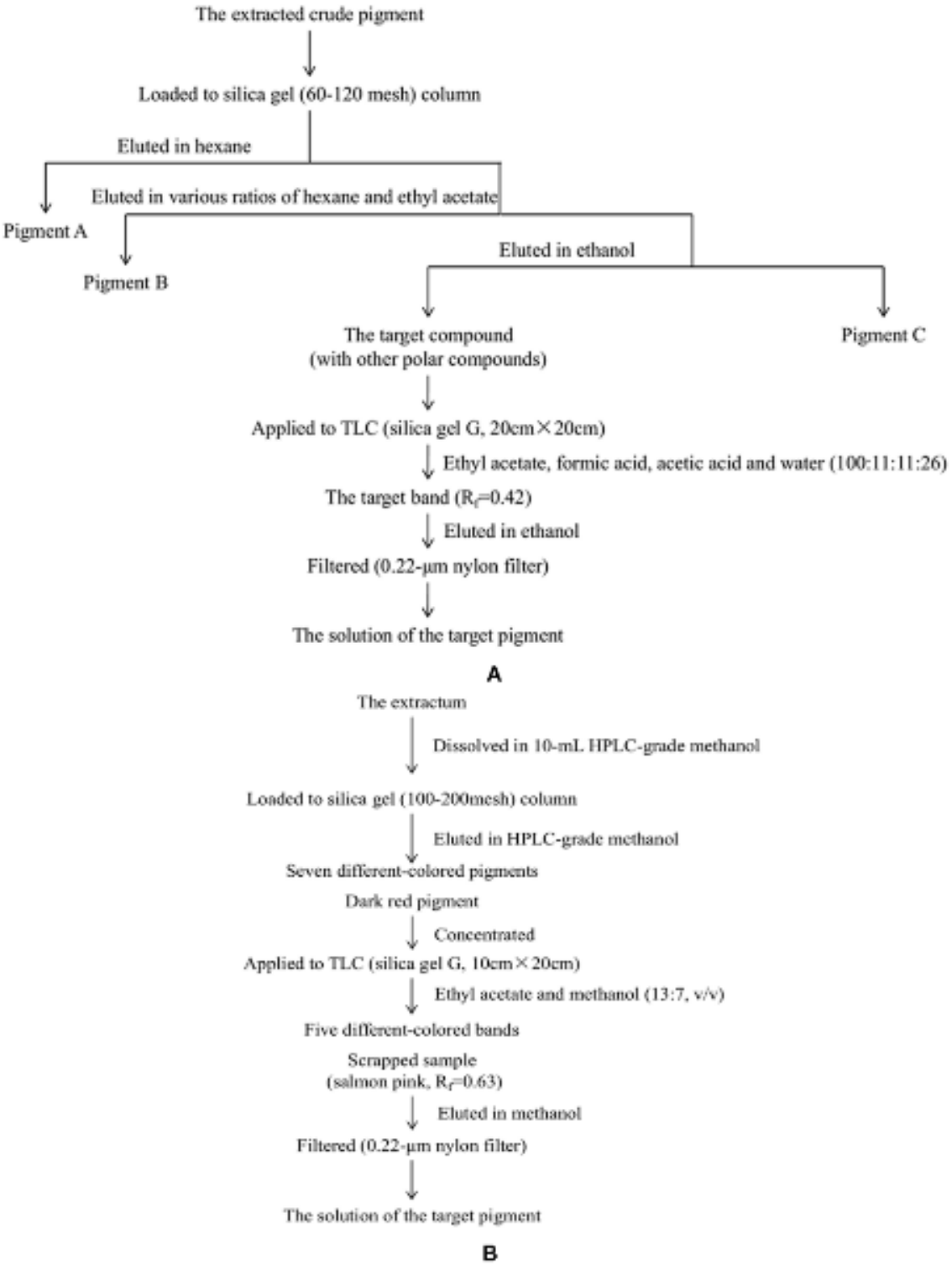

Figure 1. Workflow for the isolation and purification of red pigments from *Monascus purpureus* NFCCI 1758 (A), and *Monascus* pigments extracted from red yeast glutinous rice (B).

The aforementioned extract was dissolved in 10 mL of HPLC-grade improved eluent. Then, the solution was loaded to a silica gel (100 - 200 mesh) column, and eluted with HPLC-grade improved eluent. Seven types of pigments of different colours could be collected. A dark red pigment was chosen for further purification by TLC (silica gel G, 10 × 20 cm) following concentration. The sample was placed on the edge of a silica gel plate by means of a glass capillary (He, 2005). After these sample spots were dried, the plate was placed into a chromatographic tank with the running solvent. After 90 min, the plate was removed and dried with a hair dryer. The target band (Rf = 0.63) was scraped off and dissolved in methanol (Figure 2B). The target pigment was then separated from silica gel by filtering.

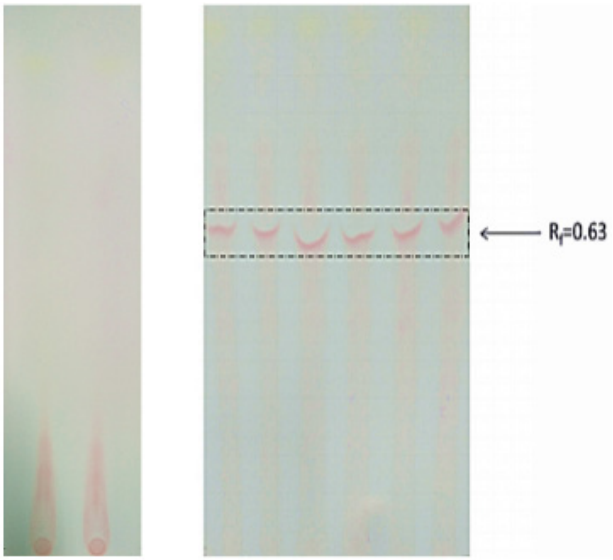

Figure 2. Thin-layer chromatogram of dark red pigments obtained by silica gel column chromatography.

Improvement of the eluent in silica gel column chromatography

In the present work, methanol (100%) was used as the eluent for silica gel column chromatography. Pandiyarajan *et al.* (2018) and Huang *et al.* (2014) performed silica gel column chromatography with chloroform:ethanol:water (1:1:1, v/v) or ethyl acetate:hexane (3:7, v/v) as eluents. A comparison was made between the three conditions.

Improvement of the running solvent in TLC

A mixture of ethyl acetate and methanol (13:7, v/v) was selected as the running solvent in the present work. In previous works (Mukherjee and Singh, 2011; Huang *et al.,* 2014; Yang *et al.,* 2018; Soumya *et al.,* 2018), ethyl acetate:acetic acid:formic acid:water (100:11:11:26, v/v), chloroform:methanol (1:1, v/v), methanol:chloroform:benzene:hexane (35:40:10:10, v/v) or ethyl acetate:*n*-hexane (3:7, v/v) were selected as running solvents to perform TLC. Their separation efficiencies were compared in the present work.

Characterisation of the target pigment

UV-vis spectroscopy of Monascus pigments

Various coloured and colourless compounds could be distinguished and identified by spectrophotometry. The maximum absorbance of the *Monascus* pigments was determined with a UV-2501pc spectrophotometer (Shimadzu, Japan), and the scanning range used was 200 - 700 nm.

High performance liquid chromatography (HPLC) analysis

HPLC analysis was conducted by a Waters e2695 separation module connected to a Waters 2489 UV/V is detector. Briefly, 20 µL of the target pigment were separated on an Agilent ZORBAX SB-Aq C_{18} column (4.6 × 250 mm, 5 µm). The mobile phase was 20% distilled water (solvent A) and 80% acetonitrile (solvent B), running at a flow rate of 0.5 mL/min, and the column temperature was set at 25°C. Prior to analysis, the mobile phase was filtered through a 0.22 µm nylon filter (ANPEL Laboratory Technologies incorporated, Shanghai, China).

Ultra-high performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-TOF-MS) analysis

The UPLC-Q-TOF-MS analysis was carried out on an Agilent 1290 Infinity II UPLC system equipped with a Jet Stream Technology ESI ion source, and an Agilent 6545 Q-TOF system (Agilent Technologies incorporated, Santa Clara, CA). Briefly, 3 µL of the target pigment were loaded on an Agilent ZORBAX SB-Aq C_{18} column (4.6 × 250 mm, 5 µm). The mobile phase was 20% distilled water (solvent A) and 80% acetonitrile (solvent B), running at a flow rate of 0.5 mL/min, and the column temperature was set at 25°C. The inspection frequency of the Q-TOF-MS system was 1700 *m/z*, and the vaporisation temperature was 320°C. Collision energies of 15, 25, and 40 eV were used in the MS/MS experiments. The ESI ion source was performed in the negative ionisation mode.

Nuclear magnetic resonance (NMR) spectroscopy

The pigment solution was dried at 45°C to obtain the red pigment residue. This residue (around 1.2 mg) was dissolved in 1 mL of CD_3OD, and analysed by NMR. The NMR spectra was recorded in CD_3OD at 20.2°C using a Bruker-ARX 400 NMR spectrometer (^1H at 300.08 MHz, and ^{13}C at 125.73 MHz) (Bruker, Billerica, MA). The following conditions were used for analysis: pulse sequences, 2 pul; pulse, 45.0 ; relax delay, 1.000 s; acq. time, 1.997 s; repetitions, 16. Chemical shifts were given in parts per million (ppm) on the delta (d) scale.

Results and discussion

Improvement of the eluent and running solvent

When chloroform:ethanol:water (1:1:1, v/v) or ethyl acetate:hexane (3:7, v/v) were used for the elution, the colour boundary of the *Monascus*

pigments was not obvious. The colour boundary was clear only with the use of methanol (100%). Moreover, the pigments could only be separated by TLC when a mixture of ethyl acetate and methanol (13:7, v/v) was used as the running solvent (Figure 2B); Otherwise, the pigments could not be further separated (Figure 2A).

Therefore, methanol (100%) and the mixture of ethyl acetate and methanol (13:7, v/v) were selected as the eluent and running solvent, respectively. Pigment separation following further purification by TLC is shown in Figure 2B. When compared with other studies (Mukherjee and Singh, 2011; Huang *et al.,* 2014; Yang *et al.,* 2018; Pandiyarajan *et al.,* 2018; Soumya *et al.,* 2018), the improved method used a lower volume of reagents and less dangerous.

Characterisation of the target pigment

UV-vis spectroscopy of Monascus pigments

The absorbance of water-soluble *Monascus* pigments is shown in Figure 3A. The wavelengths at the maximum absorption (λ_{max}) were observed at 377 and 492 nm. However, the number of components detected by HPLC was lower at 492 - 377 nm, and the target pigment was not detected at 492 nm. Therefore, 377 nm was chosen for HPLC and UPLC-Q-TOF-MS detection.

HPLC analysis

The target pigment (R_f = 0.63) obtained after TLC was filtered through a 0.22 µm nylon mesh prior to HPLC analysis. The HPLC chromatogram showed a retention time of 8.301 min for the target pigment peak in addition to a solvent peak and some impurity peaks (Figure 3B). This retention time was earlier than that of the previously reported red pigment (Mukherjee and Singh, 2011). Furthermore, when compared with the previously reported chromatogram (Yang *et al.,* 2018; Venkatachalam *et al.,* 2018), our analysis showed less impurities following the method improvement.

UPLC-Q-TOF-MS analysis

The qualitative analysis of the target pigment was performed by UPLC-Q-TOF-MS. The mass spectrum peak of the target pigment occurred at 8.386 min. The $[M-H]^-$ ion of the target pigment was 353.2116 *m/z* in the first-order mass spectrum (Figure 3C). Therefore, the molecular weight of this pigment was 354, which was consistent with the molecular weight of rubropunctatin (Figure 4A) according to the studies reported by Huang *et al.* (2014).

NMR spectroscopy

Assignments of the ^{1}H NMR and ^{13}C NMR resonances of this target pigment (R_f = 0.63) are shown in Table 1. The molecular structure of this pigment could be divided into two main parts including an azaphilone body and two following side chains: (a) the acyl five-membered chain bound to the C3; and (b) two connected unsaturated CH on the C6 endocyclic carbon atom. The ^{1}H NMR and ^{13}C NMR resonances of the azaphilone body and the two connected unsaturated CH bonds were found at the corresponding positions in previous studies (Huang *et al.,* 2014; Lian *et al.,* 2015; Venkatachalam *et al.,* 2018). Among these proton resonances, the signals of ketone C=O and ester (lactone) groups on the C13 and C2 carbon atoms were at 198.9 and 173.7 ppm in the ^{13}C NMR spectrum, respectively. The signal of carbonyl group on the C9 endocyclic carbon atoms were observed at 196.4 ppm. According to related studies (Sato *et al.,* 1997; Teng and Feldheim, 1998; Hajjaj *et al.,* 2000; Blanc *et al.,* 2010), the range of

Table 1. δ_H and δ_C of the target pigment.

Carbon	δ_H (ppm)	δ_C (ppm)	Carbon	δ_H (ppm)	δ_C (ppm)
2		173.7	C-Me9'	1.76	30.3
3		104.9	10	5.21	126
3'		174.6	11	5.08	137.3
4	6.75	99.8	12	1.07	15.6
4'		153.6	13		198.9
5	6.85	121.6	14	2.63	48.1
6		151.1	15	1.58	40
8	8.51	143.6	16	1.33	34.3
8'		119.9	17	1.25	23.7
9		196.4	18	0.86	14.7
9'		85.9			

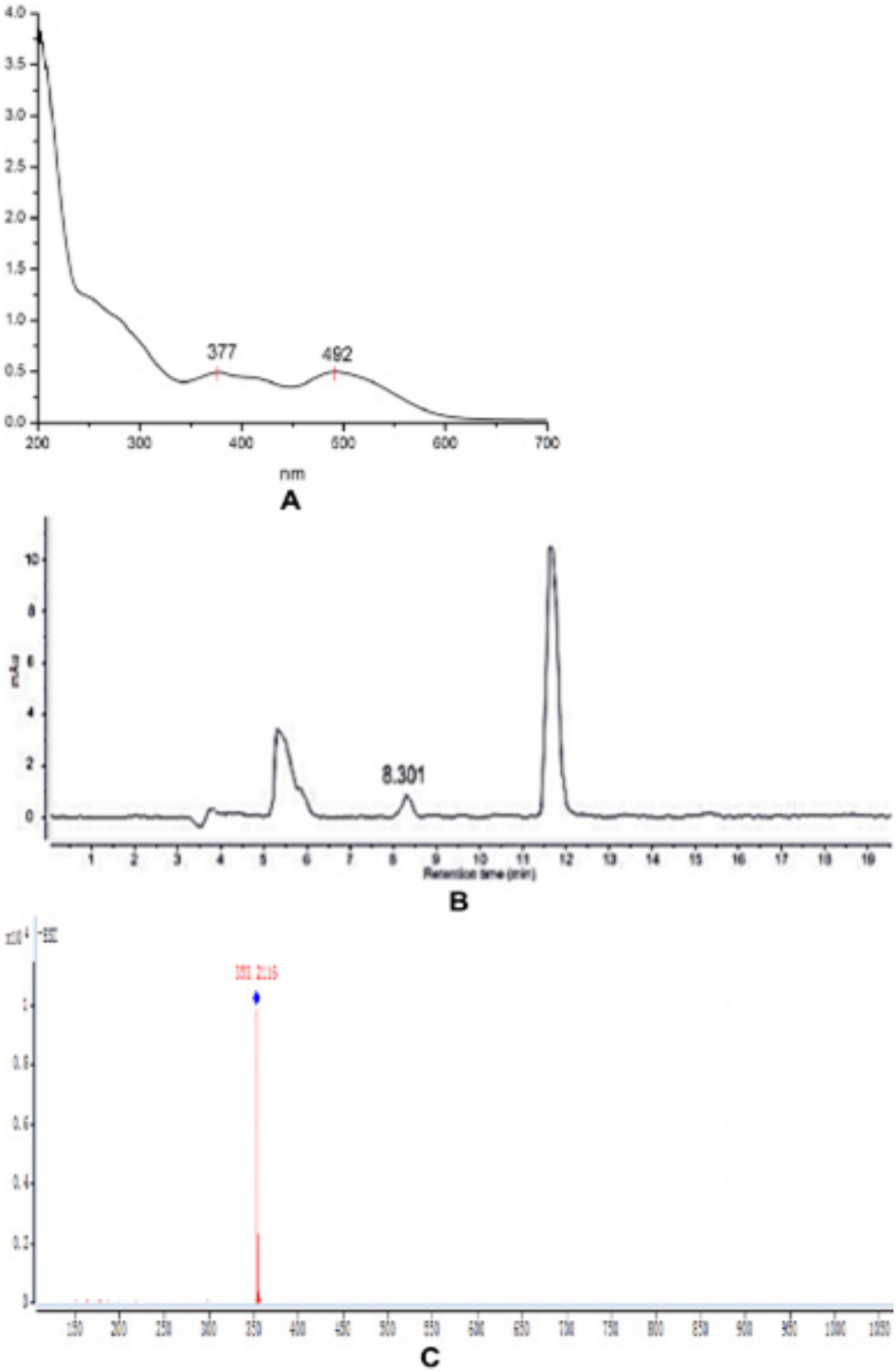

Figure 3. (A) UV-vis absorbance of *Monascus* pigments, (B) HPLC chromatogram of the target pigment, and (C) UPLC-Q-TOF-MS spectrum of the purified pigment from *Monascus*; first order mass spectrum of the target pigment (*m/z*).

the proton resonance of methyl groups was 8.0 - 30.0 ppm in the ^{13}C NMR spectrum. The proton resonances of methyl (C9', C12, and C18) were 30.3, 15.6, and 14.7 ppm (Table 1), which were consistent with those studies. Other resonances were equally well distributed.

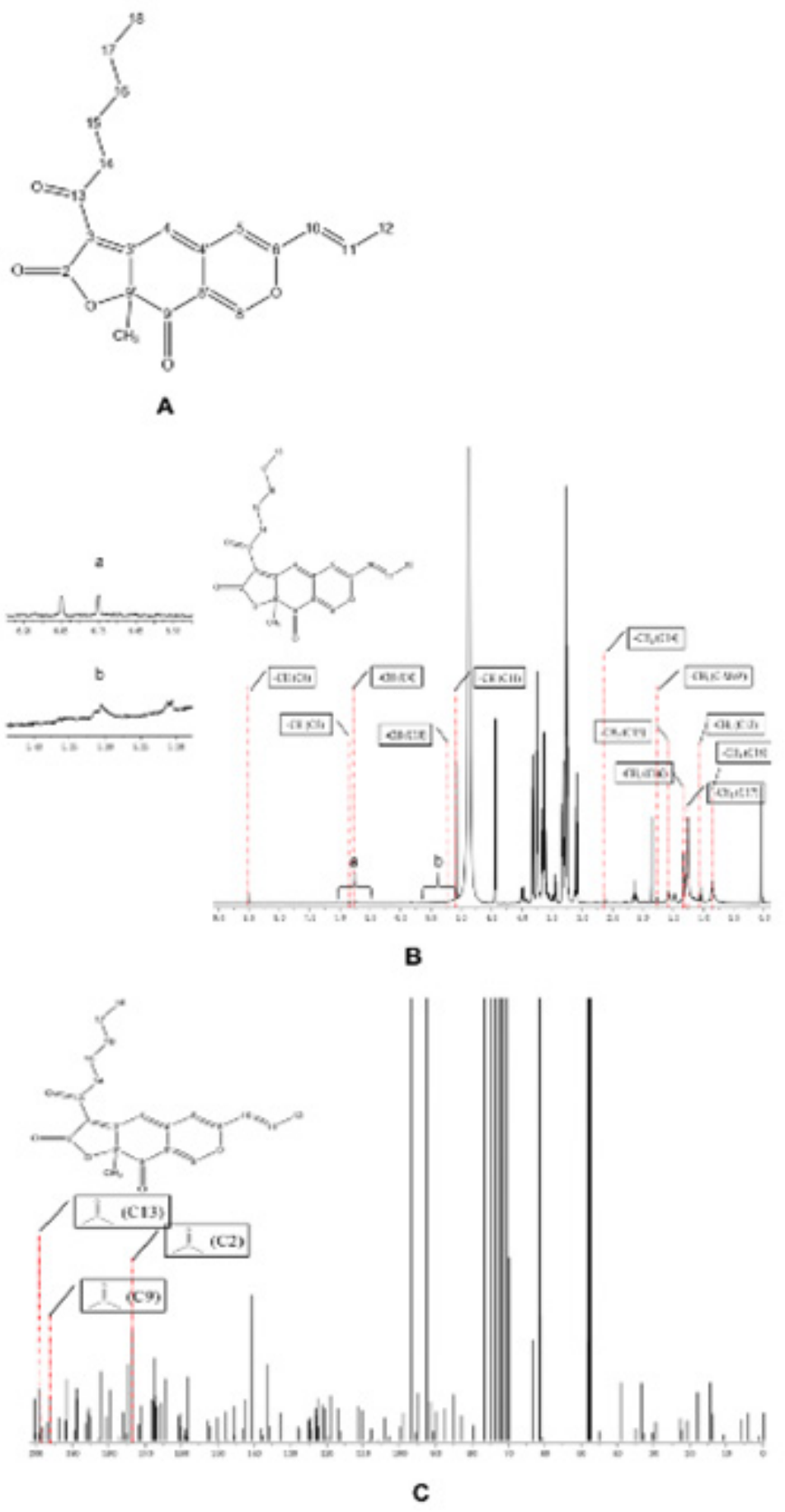

Figure 4. (A) Molecular structure of rubropunctatin, (B) ^{1}H NMR of the target pigment, and (C) ^{13}C NMR of the target pigment.

Conclusion

In the present work, the pigment obtained from red yeast glutinous rice by the improved purification method was analysed by UPLC-Q-TOF-MS and NMR. This revealed a molecular mass of 353.2 *m/z* $[M-H]^-$, and a structure comprising an azaphilone body and two following side chains: (a) acyl five-membered chain; and (b) two connected unsaturated CH. The structure of this pigment corresponded to that of rubropunctatin. Therefore, the improved method was successful to extract rubropunctatin from red yeast glutinous rice. The present work also indicated that red yeast glutinous rice can be regarded as one of the raw materials for extracting natural rubropunctatin.

Acknowledgement

The present work was financially supported by a grant from the "Twelfth Five Year Plan for Science and Technology" of Anhui Province, China (grant no.: 1401032009), the Anhui Scientific and Technical Tackle-Key-Problem Plan (grant no.: 1704a07020098), and the Colleges and Universities of Natural Science Foundation of Anhui Province (grant no.: KJ2017A127). The authors would also like to express their gratitude to EditSprings (https://www.editsprings.com/) for the expert linguistic services provided.

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