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Identification of water-soluble *Monascus* yellow pigments using HPLC-PAD-ELSD, high-resolution ESI-MS, and MS-MS

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ABSTRACT

Water-soluble *Monascus* yellow pigment (WSMYP) prepared from monascorubin is widely used in the food industry. However, the structure and composition of this pigment has been unclear until now. In the present study, the components of WSMYP has been identified by means of HPLC coupled with a photodiode-array detector (PAD) and an evaporative light scattering detector (ELSD), HPLC/ESI-MS-MS, and high resolution ESI-MS (HR-ESI-MS). Eight compounds have been separated from WSMYP and unambiguously structurally characterized; namely, monascin, ankaflavin and six novel compounds with molecular weights/structural formulae of 356/$C_{21}H_{24}O_5$, 376/$C_{21}H_{28}O_6$, 384/$C_{23}H_{28}O_5$, 404/$C_{23}H_{32}O_6$, 437/$C_{21}H_{27}NO_7S$, and 465/$C_{23}H_{31}NO_7S$. Compounds **3** (376/$C_{21}H_{28}O_6$), **7** (437/$C_{21}H_{27}NO_7S$), and **8**(465/$C_{23}H_{31}NO_7S$) are the main components in WSMYP with contents of 55.8%, 16.5%, and 10.1%, respectively. These results provide useful information for further bioactivity studies and industrial application of WSMYP.

1. Introduction

Monascus pigments (MPs) are secondary metabolites of the fungal genus, *Monascus* spp. (Patakova, 2013), which have been widely used as natural fermented food colorants in the food industry around the world, especially in China, Japan, and other southeastern Asian countries (Chen et al., 2015; Feng, Shao, & Chen, 2012). MPs also possess anticancer, antimicrobial, and various other biological activities, contributing to their wide utilization (Chen & Wu, 2016; Lee & Pan, 2012; Patakova, 2013; Vendruscolo, Tosin, Giachini, Schmidell, & Ninow, 2014; Zheng et al., 2016). Generally, MPs are mixtures of azaphilones including three main color components, i.e. *Monascus* red pigment (MRP), *Monascus* orange pigment (MOP), and *Monascus* yellow pigment (MYP). Six main compounds characterized in MPs are rubropunctamine and monascorubramine (red), rubropunctatin and monascorubrin (orange), and monascin and ankaflavin (yellow) (see Supplementary Material) (Feng et al., 2012). Recent studies have led to the identification of more than fifty further compounds in MPs (Chen & Wu, 2016). Published results have indicated that fermentation conditions affect the composition of MPs; for example, pH, carbon source, nitrogen source and temperature (Chen & Wu, 2016; Feng et al., 2012).

MYP is an important constituent of MPs. Almost half of the reported MP compounds constitute MYP (Chen & Wu, 2016). Most MYPs are hydrophobic or insoluble in water, limiting their application. Therefore, it is necessary to improve the solubility of MYP in water. Chemical modification is an effective method. Gan, Chen, and Xie (2013) and Su, Zhou, Peng, and Gan (1999), Su, Gan, and Lv (2002) prepared water-soluble MYP (WSMYP) by a chemical reaction in which MRP was reacted with Na_2S. This technique has been adopted in WSMYP production in local factories. Chen et al. (2009) evaluated the safety of the pigment and proved that no toxic effects were observed in rats continuously fed with the pigment for 90 days. However, little attention has been paid to structural study of WSMYP and its constituents are not yet clear, although some structural characterization has been performed (Su, Gan, & Lv, 2002; Su, Zhou, Peng, & Gan, 1999).

To the best of our knowledge, MPs are complex mixtures and the individual content of each compound may be low. Thus, it is very difficult to separate and obtain each single component with high purity for their analysis. LC-MS is a very convenient analytical technique for the direct and simultaneous analysis of MP components before separation and purification (Feng et al., 2012; Zheng YQ, Xin YW, & Guo, 2009). High- resolution ESI-MS (HR-ESI-MS) data permit calculation of the molecular weight, molecular formula, and degree of unsaturation of pigment components (Son et al., 2016). MS^n can generate many useful fragment ions for structural analysis of the compounds. Pigment components may be tentatively identified according to HR-MS, MS^n, and

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other information. In addition, the results of LC-MS analysis suggest that many other compounds besides those already known contribute to the color appearance (Miyake, Kong, Nozaki, & Sammoto, 2008).

The objective of the present study was to identify the main compounds of WSMYP. WSMYP was firstly separated by TLC, and then analyzed by means of HPLC-PAD-ELSD, HR-ESI-MS, and MS^2.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile for HPLC analysis were HPLC grade. All other reagents and solvents used were analytical grade, unless stated otherwise.

WSMYP was provided by Senfa Natural Pigment Co., Ltd., Nanping, Fujian province. Generally, the pigment was produced according to a procedure similar to a published process (Gan et al., 2013; Su et al., 2002). MRP was converted to WSMYP through sulfonation and alkaline hydrolysis reactions. The sulfonating agents were sodium dithionite, sulphuric acid, or other sulfo compounds.

2.2. Thin-layer chromatogram (TLC) isolation and purification

WSMYP (0.10 g) was dissolved in methanol (10 mL). The solution was centrifuged at 3000 r/min for 30 min. After centrifugation, the supernatant was recovered and separated by TLC. The mobile phase (60 mL) was a mixture of chloroform and methanol (1:1, v/v). The sample was spotted on the edge of a silica gel plate using a glass capillary (He, 2005). The silica gel plate with sample spots was placed in the mobile phase in a chromatographic tank. After 40 min, the silica gel plate was removed from the chromatographic tank, dried with a blow drier, and observed in daylight and under 254 nm UV light. R_f values of the compounds were calculated. The silica gel of the strips (R_f = 0.74 and 0.46) were scraped off and recovered. The recovered silica gel was ultrasonically extracted with methanol. After centrifugation of the extracts at 3000 r/min for 30 min, the supernatant was recovered for further analysis.

2.3. HPLC-PAD-ELSD analysis

HPLC-PAD-ELSD analysis was performed on a Waters 2695 separation module simultaneously linked to a Waters 2996 photodiode-array detector (PAD) and a Waters 2420 evaporative light-scattering detector (ELSD). The collected sample solution (10 μL) was separated on a Waters Sunfire C18 column (4.6 mm × 150 mm, 5 μm). All samples were filtered through a 0.45 μm Millipore membrane filter before injection. The mobile phase was a linear gradient of methanol (solvent A) and water (solvent B) at a flow rate of 0.8 mL/min at 30 °C, and the elution program comprised 75–85% A (0–10 min), 85–95% A (10–30 min), 95–100% A (30–35 min). The PAD was set to a scanning range of 200–700 nm. ELSD parameters were as follows: gas pressure 25 psi, temperature of sprayer 45 °C, temperature of drift tube 60 °C. Tryptanthrin was used as an internal standard to quantify the compounds.

2.4. HPLC/ESI-MS-MS and HPLC/TOF-MS analysis

The sample prepared as described in Section 2.2 was also analyzed by means of HPLC/ESI-MS-MS and HPLC/TOF-MS. The HPLC set-up was as described in Section 2.3. Spectral measurements were made at 390 nm. A step gradient elution employed methanol (solvent A) and water (solvent B) as follows: 75–83% A (0–10 min), 83–93% A (10–30 min), 93–100% A (30–35 min).

HPLC was coupled to an ion-trap mass spectrometer fitted with an ESI source (Finnigan LCQ DECA XP MAX, Thermo Fisher Co., USA) operating in positive- ion Auto MS^n mode to obtain fragment ions. The sheath gas flow and sweep gas flow velocities were 40.00 arb and 5.00 arb, respectively. The spray and capillary voltages were 4.50 kV and 10.00 V, respectively. The capillary temperature was 250.00 °C. The split ratio was 1: 4.

HPLC/TOF -MS Analysis was carried out using the same HPLC set-up equipped with an LC-time-of-flight-MS (Agilent 6210, Agilent Co., USA) fitted with an ESI source. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. All MS measurements was performed in positive-ion mode.

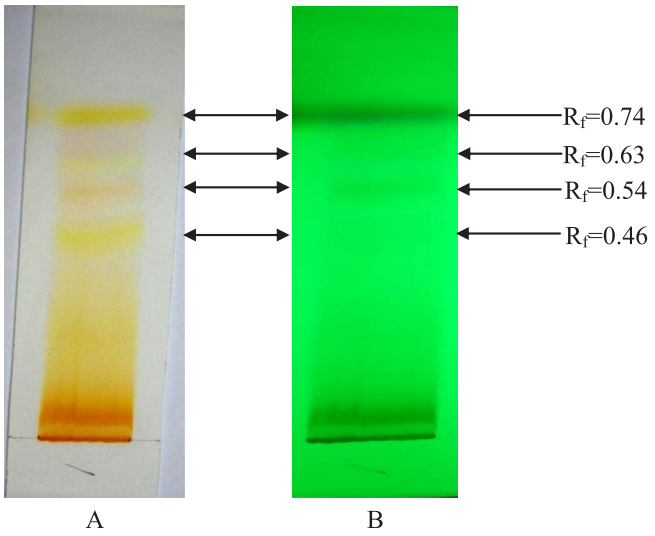

Fig. 1. Thin- layer chromatogram of water-soluble *Monascus* yellow pigments (A: Detection in daylight; B: Detection under 254-nm UV light).

3. Results and discussion

3.1. TLC isolation and purification

When observed in daylight and under 254 nm UV light (Fig. 1), four clear bands could be discerned on the silica gel plate. In daylight, two bands with R_f value of 0.63 and 0.54 were red and two bands with R_f value of 0.74 and 0.46 were yellow. Under 254 nm UV light, only the top strip (R_f = 0.74) could be seen on the plate; the other three bands were barely detectable. The two yellow bands with R_f values of 0.74 ($B_{0.74}$) and 0.46 ($B_{0.46}$) were chosen as the target groups for further analyses.

TLC is a traditional, convenient, and effective method for the isolation and purification of chemical compounds, including those of MPs (Shi et al., 2015; Xiong, Zhang, Wu, & Wang, 2015). However, compounds with similar properties cannot be fully separated by TLC. In this work, there were invariably multiple compounds in each band on the silica gel plate, necessitating further separation. Besides, the stationary phase, mobile phase, and relative humidity of the environment also affect the resolution of TLC (He, 2005).

3.2. HPLC-PAD-ELSD analysis

Compounds extracted from the chosen TLC bands were further analyzed by HPLC-PAD-ELSD. The detection wavelengths of the PAD were set at 390 and 465 nm for the detection of compounds at $B_{0.74}$ and $B_{0.46}$, respectively, corresponding to the observed λ_{max} values of the extracts dissolved in methanol. When the sample at $B_{0.74}$ was detected at a wavelength of 390 nm after separation on the column, six peaks were resolved (Fig. 2A). They appeared in two groups of peaks with retention times (t_R) around 8 min and 12 min, respectively. They were numbered as Peak **1–6** according to their elution order. Peaks in the