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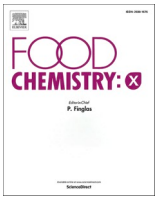

Precursor-directed production of water-soluble red *Monascus* pigments with high thermal stability via azaphilic addition reaction-based semi-synthesis

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ABSTRACT

Red *Monascus* pigments (MPs) are a large group of polyketides from the fungus *Monascus* which have been widely used as food colorants. In this study, a variety of red MPs congeners were prepared to explore promising water-soluble candidates for application in liquid food formulations. The results showed that by combining the two-stage, low-pH fermentation strategy with a downstream purification step of fractional crystallization, precursors of red MPs, namely monascorubrin and rubropunctatin, were obtained with a purity of 91.9%. Then, via the azaphilic addition reaction, 18 types of red MPs congeners carrying different amino acid moieties (MPs-aa) were semi-synthesized. Compared to rubropunctamine and monascorubramine, the water solubility, pH and thermal stability of MPs-aa were improved greatly. MPs-His, MPs-Phe, MPs-Tyr and MPs-Trp were identified to be the most resistant to pasteurization. These findings provide water-soluble red MPs candidates with high thermal stability and an attractive approach for their large scale production.

1. Introduction

Monascus pigments (MPs) are a group of polyketides produced by the filamentous fungus *Monascus*, and have been used as natural food colorants for processed meats (e.g. sausages and hams), marine products (e.g. fish paste and surimi), fermented bean curd, red rice wine, tomato ketchup and so on (Jung, Choe, Nam, Cho, & Shin, 2011; Chen et al., 2017). Nowadays, more than 110 compounds of MPs have been reported, and their biogenesis was deduced to be related to a unitary polyketide biosynthetic pathway with a couple of synthetic branches (Chen, Feng, Molnar, & Chen, 2019; Pavesi, Flon, Mann, Leleu, Prado, & Franck, 2021). The biosynthesis of MPs compounds occurs in pairs, involving the incorporation of a β-ketooctanoic or β-ketodecanoic acid moiety (Fig. S1). This is attributed to the flexible control of chain length by the specialized fatty acid synthetase responsible for the side chain fatty acyl moiety (Chen et al., 2017). Based on their maximum absorption wavelengths (λ_{max}), these pigment compounds are classified into three categories: orange, red and yellow (Chen et al., 2017). The six major pigment components in the conventional colorants of MPs, also known as Hong Qu, red koji, red yeast rice, or red fermented rice, were previously found to be the orange pair of rubropunctatin (Haws, Holker, Kelly, Powell, & Robertson, 1959) and monascorubin (Nakanishi, Ohashi, Kumasaki, & Yamamura, 1962), the yellow pair of monascin (Fielding, et al., 1961) and ankaflavin (Manchand, Whalley, & Chen, 1973), and the red pair of rubropunctamine and monascorubramine (Hiroi, Shima, Isobe, & Kimura, 1975) (Fig. S1). Due to the lipophilic nature of the six major pigment compounds, the application of conventional MPs colorant in liquid formulations like carbonated beverages and canned fruit juices is limited (Wong & Koehler, 1983; Lin, Yakushijin, Büchi, & Demain, 1992).

In the latter part of the 1970s and early 1980s, several chemical techniques for improving the water solubility of red MPs were patented (Lin et al., 1992). These techniques involved chemical reactions between lipophilic orange MPs and hydrophilic proteins, peptides, amino acids, amino sugars, amino alcohols, chitosan, nucleotides, or nucleic acids. This reaction mechanism is now known as that, the orange MPs rubropunctatin and monascorubin are aminophilic and readily react with amino group ($-NH_2$) via the azaphilic addition reaction, giving rise to compounds in which the pyran moiety changed into the *N*-substituted dihydropyridine moiety (Fig. S2) (Liu & Wang, 2022). Based on this principle, MPs containing *N*-substituted dihydropyridine moieties were successfully produced via supplementing an excess of primary amines,

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particularly amino acids, during submerged fermentation (Lin et al., 1992; Jung, Kim, Kim, & Shin, 2003; Jung, Kim, & Shin, 2005; Kim, Jung, Kim, & Shin, 2006; Jeun, Jung, Kim, Yong, Youn, & Shin, 2008; Kim, Kim, Jeun, Choi, & Shin, 2010; Hajjaj, Francois, Goma, & Blanc, 2012; Liu, Zhang, & Wang, 2021). In recent decades, researchers at Yonsei University in Korea have conducted substantial investigations on MPs carrying an amino acid moiety (designated MPs-aa in the following text). They found that, in addition to the improved solubility, some MPs-aa exhibited several other advantages over the conventional red MPs rubropunctamine and monascorubramine, including enhanced photostability (e.g. MPs-Asp, MPs-Gly, MPs-Ile, MPs-Phe and MPs-Thr) (Jung et al., 2005), and multiple biological activities such as lowing hyperlipidemia (e.g. MPs-Thr) (Jeun et al., 2008), antiobesity (e.g. MPs-Trp and MPs-Thr) (Kim et al., 2010; Nam, Choe, & Shin, 2014, Choe, Jung, Kim, Shin, Johnston, & Ku, 2020; Choe et al., 2020), antimicrobial activity (e.g. MPs-Phe and MPs-Tyr) (Kim et al., 2006) and so on. The exceptional characteristics of MPs-aa made them highly appealing as natural food colorants, as well as functional supplements.

In practical applications, MPs are commonly employed in a non-separated mixture state, and the constituents of industrially produced MPs are rarely revealed. Since it has been found that the physical–chemical and biological properties of MPs-aa are dependent on their chemical structure, there is a requirement to produce highly pure MPs-aa carrying a distinct amino acid moiety for different purposes. Although supplementing a specific amino acid to submerged culture has been found to lead to the accumulation of the desired MPs-aa, a significant amount of MPs impurities usually produced simultaneously, including yellow MPs monascin and ankaflavin, red MPs rubropunctamine and monascorubramine, and various MPs derivatives formed through azaphilic addition reactions with complex primary amines involved in microbial metabolism (Jung et al., 2003; Liu et al., 2021). As a consequence, the composition of the MPs-aa product was complex and unpredictable. Moreover, amino acids could serve as a nitrogen source in fermentation processes, and exerted complicated impacts on the proliferation of *Monascus* and the biosynthesis of polyketides. Valine, leucine, and methionine were shown to facilitate effective growth; however, they led to poor production of MPs-aa. On the other hand, glutamate, alanine, and proline were observed to stimulate the production of MPs-aa, but also be associated with elevated levels of the mycotoxin citrinin (Hajjaj et al., 2012; Yin, Yang, Zhu, & Huang, 2022). In contrast, the employment of *in vitro* chemical modification via azaphilic addition reaction seems to be a more effective strategy, which facilitates the smooth transformation of rubropunctatin and monascorubin into desired MPs-aa under mild conditions (Liu & Wang, 2022). However, none of the MPs-aa has been produced on large-scale due to the difficulty in preparing a sufficient amount of pure monascorubrin and rubropunctatin, which are indispensable precursors for the *in vitro* transformation.

In the present study, a large amount of rubropunctatin and monascorubin with high purity were obtained by combining an efficient orange MPs fermentation method with a one-step crystallization purification process. The purified rubropunctatin and monascorubin were then used as precursors for semi-synthesis of various MPs-aa via the azaphilic addition reaction-based chemical modification, and their solubility and colorfastness under various pH and temperature conditions were investigated. These results provide guidance for the efficient production of desired water-soluble red MPs-aa with enhanced stability.

2. Materials and methods

2.1. Microorganism

M. ruber M7 (CCAM 070120, Culture Collection of State Key Laboratory of Agricultural Microbiology, which is part of China Center for Type Culture Collection (CCTCC), Wuhan, China) was a model strain used to study the MPs biosynthesis pathway (Chen et al., 2017). The strain was maintained on PDA (potato dextrose agar, potato 200 g, glucose 20 g, and agar 15 g, per liter of tap water) slants at 4 °C.

2.2. Fermentation of rubropunctatin and monascorubin

The orange MPs rubropunctatin and monascorubin, which are precursors for the azaphilic addition reaction-based semi-synthesis of red MPs, were produced using the two-stage, low-pH fermentation method described in our previous study (Li et al., 2019). The initial fermentation stage was conducted at an optimal pH for promoting the substantial proliferation of mycelia; while the second fermentation stage was conducted under an extremely acidic condition of pH3.0 to facilitate the extensive accumulation of rubropunctatin and monascorubin. In brief, a 0.2-mL freshly harvested spore suspension (10^5 spores/mL) of *M. ruber* M7 was inoculated in a 500-mL Erlenmeyer flask with 100 mL PDB medium containing 0.2 % (m/v) 80–100 mesh rice powder and incubated in IS-DRS3 stackable incubator shakers (Crystal Technology & Industries, Inc. Dallas, USA) at 28 °C, 200 rpm for 40 h when large quantities of mycelia without visible pigments were formed; then an equal volume of 0.1 mol/L citric acid-phosphate buffer at pH 3.0 was added, and cultivation was continued for 5 additional days.

2.3. Extraction and purification of rubropunctatin and monascorubin

The fermentation broth was filtered through Whatman filter paper (No. 4). The resulting filter cake was resuspended in an ethanol aqueous solution (70 %, v/v, pH 2.0 adjusted with formic acid), homogenized using the FSH-II high speed homogenizer (Jiangsu Huanyu Scientific Instrument Co., Ltd, Changzhou, China) and extracted for 12 h at 4 °C in dark. Subsequently, the undissolved culture debris were removed by filtration with Whatman filter paper (No. 4), and the filtrate was collected as crude MPs extract.

The concentration of crude MPs extract was addjusted to an $OD_{470\ nm}$ of 45.0 ± 1.0 with 70 % ethanol aqueous solution (pH 2.0 adjusted with formic acid). Then, orange MPs rubropunctatin and monascorubin were crystallized from the crude MPs extract by adding 0.5 vol of acidic water (pH 2.0 adjusted with formic acid) and keeping at −20 °C for 2 h. Subsequently, the orangish red crystal particles were harvested by filtration through Whatman filter paper (No. 4), washed with acidic water (pH 2.0 adjusted with formic acid), dried by hot air drying at 40 °C and stored at −20 °C in dark.

2.4. Production of red Monascus pigments with an amino acid moiety

In vitro chemical modification was performed in a 50 % (v/v) methanol aqueous solution containing 0.1 mol/L phosphate buffer at pH 7.0. A reaction mixture of 100 mL contained 12 mg of the purified orange MPs rubropunctatin and monascorubin and 8 mmol/L (final concentration) amino acid. The reaction mixture was incubated at 30 °C, 250 rpm for 2 h, and then desalted and concentrated using C18 SPE columns. After washing with deionized water, the red MPs-aa absorbed in C18 SPE columns were eluted by a 90 % (v/v) methanol aqueous solution. The MPs-aa elute was vacuum dried at 40 °C to yield a red solid powder. Amino acids used in the reactions included 18 kinds of natural proteinogenic amino acids, namely L-arginine (Arg), L-lysine (Lys), L-asparagine (Asn), L-glutamate (Glu), L-glycine (Gly), L-alanine (Ala), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-methionine (Met), L-phenylalanine (Phe), L-tyrosine (Tyr), L-tryptophan (Trp), L-threonine (Thr), L-serine (Ser), L-aspartate (Asp), L-histidine (His) and L-glutamine (Gln).

Instead of 8 mmol/L amino acid, 1 mol/L ammonia was added to the reaction mixture to produce the conventional red MPs rubropunctamine and monascorubramine under the same condition.

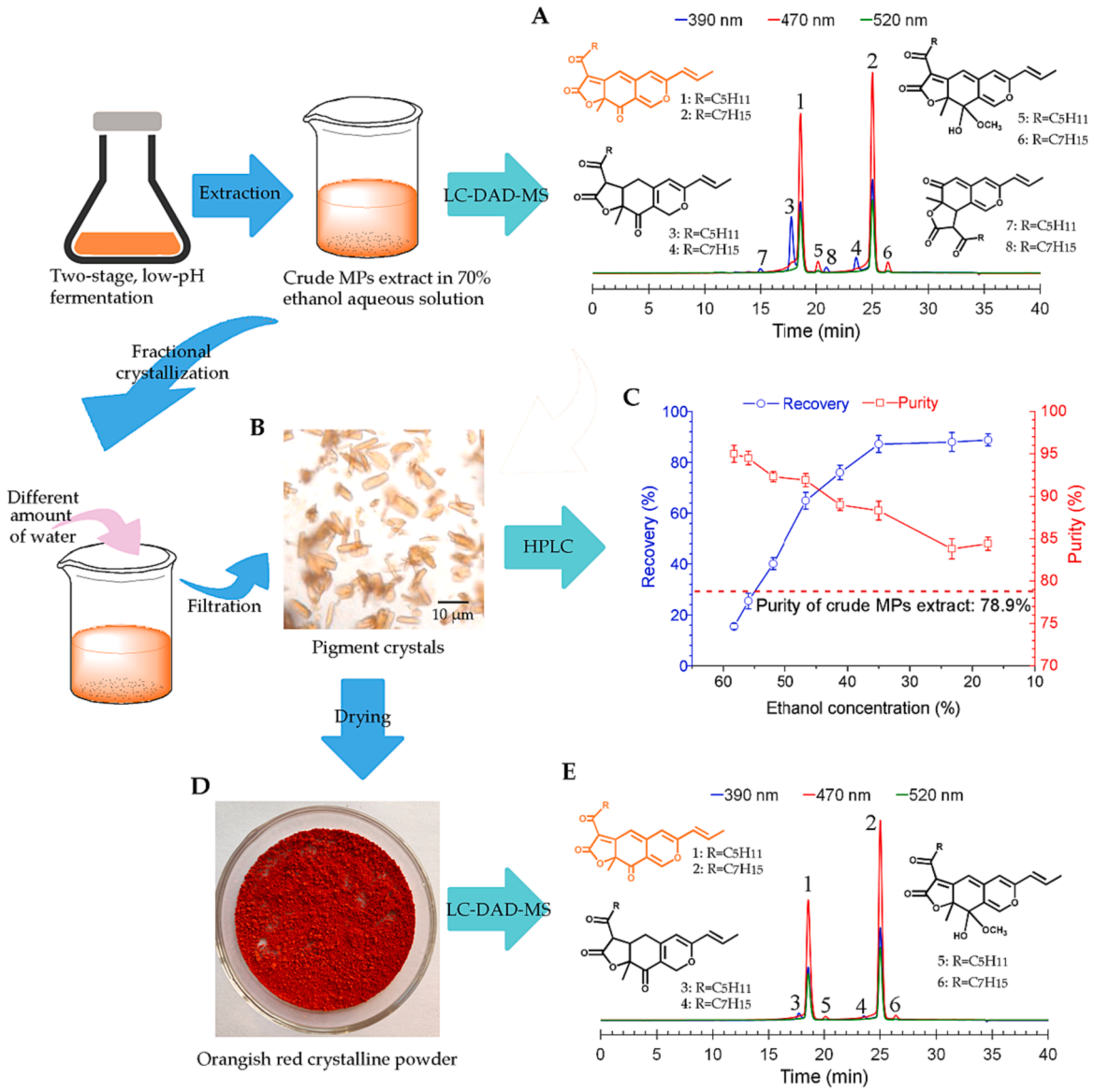

Fig. 1. Preparation of precursors for red MPs. (A) HPLC profile of the crude MPs extract. (B) The MPs crystals observed under a microscope. (C) The recovery and purity of the precursors obtained under different crystallization condition. Purity of the precursors in the crude MPs extract was 78.9% (32.2% rubropunctatin and 46.7% monascorubrin). (D) The powder of the dried precursors. (E) HPLC profile of the precursors yield from fractional crystallization. HPLC peak 1 to 8 corresponded to rubropunctatin, monascorubrin, monascin, ankaflavin, monasphilol-methoxy A, monasphilol-methoxy B, monasfluore A and monasfluore B, respectively.

2.5. Water solubility analysis

A quantity of 250 mg of MPs-aa powder was dissolved individually in 10 mL of deionized water at 25 °C with ultrasonic assistance. The undissolved pigment residuals were collected by centrifugation (10000 g, 20 min), vacuum dried and weighed to determine the dissolving capacity of the MPs-aa in pure water.

2.6. pH treatment and visual color difference evaluation

MPs-aa was individually dissolved to an $OD_{\lambda max}$ of 2.0 ± 0.1 in a 40 % methanol aqueous solution. The MPs-aa solution was mixed with an equal volume of 0.1 mol/L citric acid-phosphate buffer (pH 3.0, pH 5.0 or pH 7.0), sodium carbonate-sodium bicarbonate buffer (pH 9.0) or borax-sodium carbonate buffer (pH 11.0), and kept in dark at 25 °C for 24 h. The color characteristics of MPs-aa at different pH values were evaluated visually and instrumentally using a TU-1900 UV–Vis spectrophotometer (Presee, Beijing, China). The conventional red MPs rubropunctamine and monascorubramine were used as the control, and were treated under the same condition.

2.7. Heat treatment and thermal stability evaluation

To evaluate the thermal stability of MPs-aa at different pH conditions, the above MPs solutions at pH 3.0, pH 5.0, pH 7.0 and pH 9.0 were kept from light at 80 °C for 6 h, and the remaining MPs-aa were measured at their λ_{max}. Remaining pigments (%) = $A / A_0 \times 100$ %, where A_0 and A were absorbance at λ_{max} before and after heating, respectively. The conventional red MPs rubropunctamine and monascorubramine were used as the control, and were treated under the same condition.

2.8. Degradation kinetic analysis in the model beverage during pasteurization

The model beverage system was prepared according to Karangutkar

& Ananthanarayan (2021) in which McIlvaine buffer solution (0.1 mol/L citric acid-sodium citrate) at pH 5.0 was used. The formulation consisted of 13 % (w/v) sucrose, 0.05 % (w/v) sodium benzoate and appropriate amount of pigments to give a similar visual color appearance (Fig. S3). The commercial anthocyanins-based water-soluble food colorant of grape skin extract (GSE, INS 163ii) (Zhejiang Yinuo Biotechnology Co., Ltd, Yiwu, China) was used as the control. Prepacked beverages were pasteurized using water bath at temperatures of 65 °C, 75 °C, 85 °C and 95 °C, respectively. The thermal stability was expressed in terms of rate constant (k) and half-life value ($t_{1/2}$), calculated using regression analysis of ln (A/A_0) versus heating time (t), where A_0 and A were absorbance at λ_{max} before and after heating for the time of t, respectively.

2.9. UV–vis spectroscopic analysis of pigments

Ultraviolet–visible (UV–Vis) spectroscopic analysis was performed as described by Li et al. (2019). The absorbance spectrum of MPs-aa was recorded using a TU-1900 UV–Vis spectrophotometer (Presee, Beijing, China) in a range of 350 to 550 nm at 2-nm intervals. The optical density (OD) units at λ_{max} were used as an index of the concentration of pigments.

2.10. HPLC analysis of pigments

HPLC analysis was performed using a reverse-phase C18 column (Inertsil ODS-3, 250 × 4.6 mm, 5 μm; Shimadzu, Kyoto, Japan) with a flow rate of 0.8 mL/min, in an Agilent 1200 HPLC system (Agilent, California, USA) equipped with a diode array detector (DAD; Agilent, California, USA). The mobile phases were acetonitrile (A), water (B), and water at pH of 3.0 (C). Gradient elution was performed as follows: solvent B was maintained at 5 % (v/v) throughout; step gradient for solvent A was 55 % (v/v) to 65 % (v/v) in 3 min; 65 % (v/v) to 90 % (v/v) in 22 min; 90 % (v/v) for 5 min; 90 % (v/v) to 55 % (v/v) in 1 min; 55 % (v/v) for 9 min. The column temperature was kept at 30 °C. The detection wavelength was 390 nm for yellow MPs, 470 nm for orange MPs and 520 nm for red MPs.

2.11. LC-DAD-MS analysis of pigments

LC-DAD-MS was performed with a AB Sciex TripleTOF 5600$^+$ LC/MS system (AB Sciex, Framingham, USA), using a Kinetex 2.6 μm F5 column (100 mm × 2.1 mm; Phenomenex, Torrance, USA) with a flow rate of 0.2 mL/min. The mobile phase consisted of water (A, with 0.1 % formic acid) and acetonitrile (B). Gradient elution was performed as follows: 0–1 min, 95 % (v/v) A; 1–8 min, 95 % (v/v) A to 15 % (v/v) A; 8–9 min, 15 % (v/v) A; 9–9.1 min, 15 % A (v/v) to 95 % (v/v) A; 9.1–11 min, 95 % (v/v) A. Ten microliters of each sample was injected into the column. The temperatures of column and samples were held at 30 °C and 4 °C, respectively. The detection wavelength of DAD detector was from 210 to 600 nm. Mass spectrometry (MS) was performed using a triple quadrupole detector equipped with an electrospray ionization (ESI) source in positive ionization mode. The scan range was *m/z* 100–1000. Ionization spray voltage was set to 5500 V. Sheath gas was 379 kPa, auxiliary gas was 379 kPa, and curtain gas 172 kPa. Temperature of ESI source was 550 °C.

2.12. Statistical analysis

All tests were performed in triplicate. The results were expressed as the mean values ± standard deviation (SD). The data were submitted to analysis of variance (ANOVA), and significance of differences was determined by Duncan's multiple range tests where necessary. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation of precursors for red MPs

During the azaphilic addition reaction-based semi-synthesis of red MPs, the orange MPs monascorubrin and rubropunctatin serve as indispensable precursors. It has been demonstrated that monascorubrin and rubropunctatin were accumulated under acidic conditions due to the activation of genes involved in their biosynthesis, as well as the blocking of the azaphilic addition reaction in fermentation medium (Li et al., 2019). Thus, the two-stage, low-pH fermentation strategy developed by Li et al. (2019) was employed for production of monascorubrin and rubropunctatin. LC-DAD-MS analysis showed that there were 8 pigment components in the crude MPs extract, and the two dominant ones were rubropunctatin and monascorubrin, accounting for 32.2 % and 46.7 %, respectively (Fig. 1A, Fig. S4 and S5). The impurities included four yellow MPs, namely monascin, ankaflavin, monasfluore A and monasfluore B, and two orange MPs, namely monasphilol-methoxy A and monasphilol-methoxy B (Fig. 1A, Fig. S4 and S5).

According to the calculated values of octanol/water partition coefficient (LogP) and aqueous solubility (LogS), all the eight pigment components in the crude MPs extract showed high lipophilicity, with LogP values ranging from 2.10 and 3.86, and poor water solubility, with LogS values ranging from −5.06 to −3.38 (Table S1). Whereas, variations in lipophilicity were observed between the precursors and impurities. Three of the impurities, namely monascin, monasfluore A and monasphilol-methoxy A, had much lower LogP values than both of rubropunctatin and monascorubrin, and the rest impurities, namely ankaflavin, monasfluore B and monasphilol-methoxy B had much lower LogP values than monascorubrin. Based on these differences, fractional crystallization was then carried out to separate rubropunctatin and monascorubrin from the crude MPs extract.

Upon the addition of 0.2 to 3.0 volumes of water (pH 2.0) to the ethanolic extract of crude MPs, its concentration of ethanol decreased from the original 70.0 % to 17.5 %, and the formation of orangish red crystals was observed (Fig. 1B). As shown in Fig. 1C, as the ethanol concentration dropped, more and more pigment crystals were recovered, but the purity of rubropunctatin and monascorubrin gradually decreased. When the ethanol concentration fell to a range of 46.7 %–58.3 % by adding 0.2–0.5 volumes of acidic water, the purity of rubropunctatin and monascorubrin experienced an increase from the initial 78.9 % (consisting of 32.2 % rubropunctatin and 46.7 % monascorubrin) to a range of 92 %–95 % (Fig. 1C). Considering the recovery efficiency, reducing the ethanol concentration to 46.7 % by adding 0.5 volumes of water was the optimal condition for the fractional crystallization of rubropunctatin and monascorubrin. Using this developed purification approach, an orangish red crystalline powder with 91.9 % precursor MPs (consisting of 36.0 % monascorubrin and 55.9 % rubropunctatin) was obtained (Fig. 1D and E). When dissolved in methanol, an orange solution with an absorbance of 1.0 at 470 nm was determined to be equivalent to 14.35 mg/L of the purified crystalline pigment powder, accounting an extinction coefficient (ε_{max}) about 2.6×10^4 L/(mol·cm).

To date, the separation and purification of rubropunctatin and monascorubrin have basically relied on chromatography-based techniques, including silica-gel column chromatography (Wong & Koehler, 1983; Gu, Xie, Zhang, & Wang, 2019), preparative silica gel thin layer chromatography (TLC) (Lin et al., 1992), resin-based column chromatography (Chen et al., 2021) and preparative HPLC (Jia et al., 2019). These chromatographic techniques were only suitable for small-scale laboratory sample preparation due to their high cost and low efficiency. Here, combining the two-stage, low-pH fermentation method with the one-step crystallization purification process, (0.79 ± 0.04) g of pigment powder with 91.9 % of rubropunctatin and monascorubrin were obtained from 1 L of fermentation medium, suggesting that it is a promising strategy for the efficient preparation of rubropunctatin and

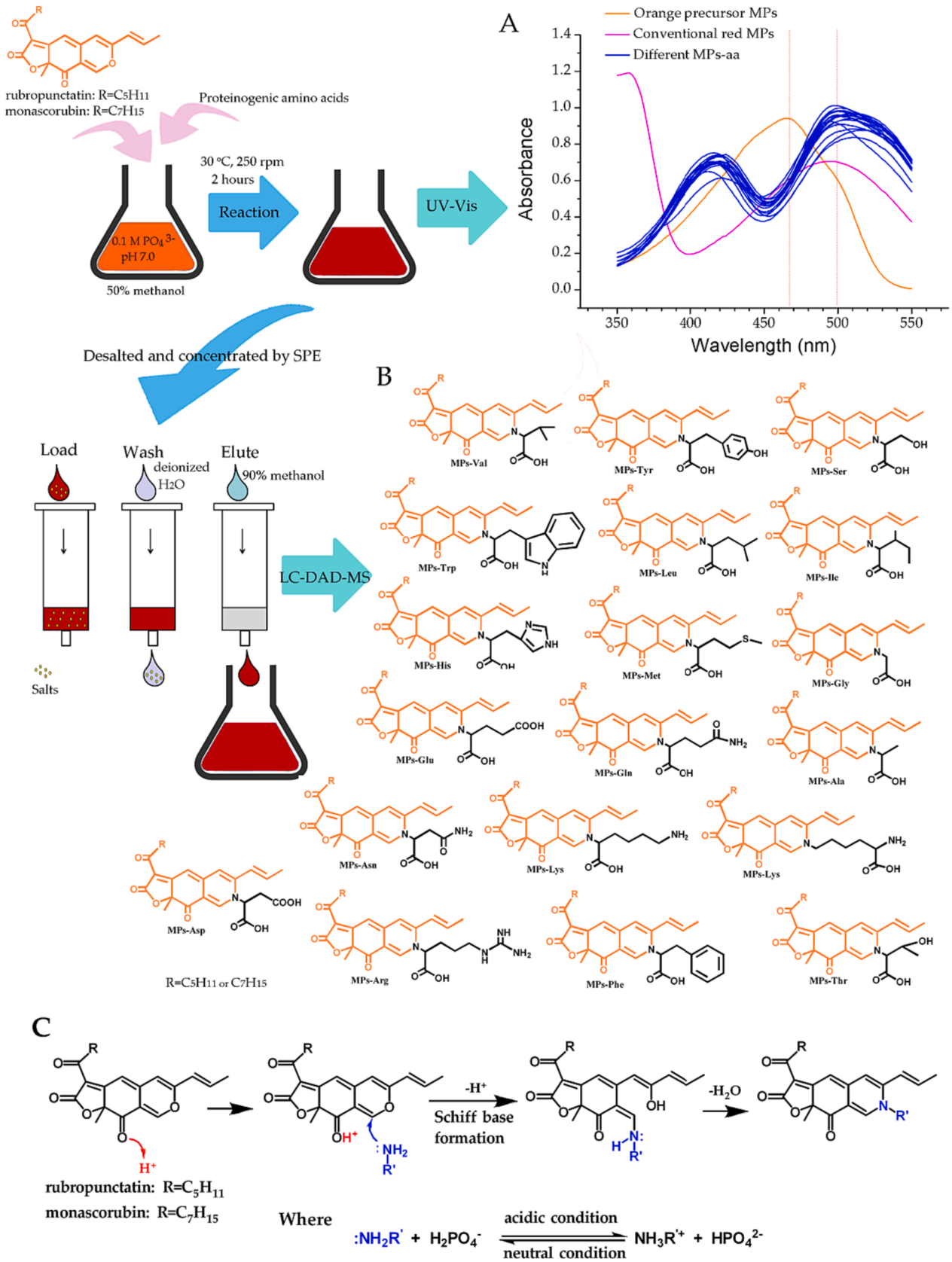

Fig. 2. Semi-synthesis of MPs-aa via azaphilic addition reaction. (A) The UV–Vis absorption spectra of the MPs-aa products. (B) The predicted structures of 18 types of MPs-aa. (C) A proposed pathway for azaphilic addition reaction of rubropunctatin and monascorubin with amino group. $H_2PO_4^-$ acting as Brønsted acid catalyst provides a proton (H^+) (red) to activate rubropunctatin and monascorubin. NH_2R' with a lone pair of electrons (blue) attacks the activated azaphilone smoothly (Liu & Wang, 2022).

Table 1
Spectral characteristics and water solubility of MPs-aa.

Pigments	λ_{max} (nm)[a]	ε_{max} [10^3 L/(mol·cm)][a]	Water solubility g/L	Water solubility $OD_{\lambda max}$[b]
MPs-Lys	502	14.2 ± 0. 1	> 25.0	>671.3 ± 4.7
MPs-Asn	502	15.1 ± 0. 3	> 25.0	>781.4 ± 14.3
MPs-Glu	504	14.2 ± 0. 4	> 25.0	>685.3 ± 19.3
MPs-Gly	516	13.8 ± 0.3	> 25.0	>757.4 ± 16.5
MPs-Ala	512	13.7 ± 0.0	> 25.0	>711.2 ± 2.1
MPs-Val	500	15.0 ± 0.1	> 25.0	>795.4 ± 5.3
MPs-Leu	500	15.1 ± 0. 1	> 25.0	>780.8 ± 5.2
MPs-Ile	500	15.1 ± 0. 2	> 25.0	>760.2 ± 9.7
MPs-Met	500	15.7 ± 0.4	> 25.0	>760.2 ± 19.4
MPs-Phe	498	15.7 ± 0.5	> 25.0	>724.0 ± 23.1
MPs-Tyr	500	15.2 ± 0.0	> 25.0	>703.0 ± 1.9
MPs-Trp	504	14.7 ± 0.2	> 25.0	>637.6 ± 8.7
MPs-Ser	504	14.7 ± 0.1	> 25.0	>737.5 ± 5.0
MPs-Thr	510	15.3 ± 0.3	> 25.0	>783.3 ± 15.4
MPs-Asp	510	12.3 ± 0.6	> 25.0	>686.4 ± 33.5
MPs-His	494	14.3 ± 0.1	> 25.0	>679.8 ± 4.8
MPs-Gln	502	15.1 ± 0.3	> 25.0	>733.9 ± 14.6
MPs-Arg	502	15.2 ± 0.2	2.1 ± 0.7*	57.8 ± 8.7
Conventional red MPs	494	6.0 ± 0.4	3.9 ± 0.2*	52.0 ± 2.8

Data are presented as mean ± standard deviation (n = 3).
[a] Wavelength of maximum absorbance (λ_{max}) and maximum molar extinction coefficient (ε_{max}) were measured in the 50% methanol-phosphate buffer at pH 7.0.
[b] For the conventional red MPs (the control) and MPs-Arg, $OD_{\lambda max}$ values of the saturated aqueous solution were shown; for the other MPs-aa, $OD_{\lambda max}$ values of aqueous solution at 25.0 g/L were shown.

monascorubrin in a large scale. Based on the most recent knowledge, this is the simplest, most convenient, and least expensive approach for the preparation of highly pure rubropunctatin and monascorubrin, which are indispensable precursors for the semi-synthesis of MPs-aa. The adequate preparation of pure monascorubrin and rubropunctatin is crucial in enabling the efficient production of MPs-aa by semi-synthesis on a large scale, hence facilitating extensive investigation into their properties and potential applications.

3.2. Semi-synthesis of MPs-aa via azaphilic addition reaction

Upon reacting with ammonia or 18 natural L-amino acids, the orange precursor MPs (with a λ_{max} around 470 nm) were successfully converted in to red ones, with a λ_{max} around 500 nm (Fig. 2A, Table 1). LC-DAD-MS analysis revealed that all the azaphilic addition reactions were virtually complete and no by-products formed (Fig. S6). These results suggested that 18 types of MPs-aa, along with the conventional red MPs rubropunctamine and monascorubramine, were successfully synthesized via the azaphilic addition reaction, including MPs-Gln which were failed to be produced by Gln-supplementing fermentation method previously (Jung et al., 2003). However, two natural proteinogenic amino acids, namely L-proline and L-cysteine were unable to generate the corresponding pure MPs-aa in this study. This was found to be attributed to that L-proline is a secondary amine lacking the reactive $-NH_2$ group, while L-cysteine was quickly oxidized in the absence of air protection at neutral pH condition.

As shown in Table 1, the ε_{max} values for all the MPs-aa fell within the range of 12.3 × 10^3 and 15.7 × 10^3 L/(mol·cm), whereas that of the conventional red MPs rubropunctamine and monascorubramine was more than 2 times lower, approximately being 6.0 × 10^3 L/(mol·cm). These results indicated that the pigment solution intensity of all the MPs-aa was significant greater than that of rubropunctamine and monascorubramine. Therefore, to achieve a similar color appearance in food products, the utilization of MPs-aa would lead to a lower dosage requirement as compared to the conventional red MPs.

3.3. Water solubility of MPs-aa

Water solubility of MPs-aa is an essential property in aspect to their application in aqueous system like beverage. As shown in Table 1, except for MP-Arg, the water solubility of all the MPs-aa was improved greatly, increasing at least by 5.4 times when compared to the conventional red MPs rubropunctamine and monascorubramine. At the concentration of 25.0 g/L, the MPs-aa solution was dark red, with $OD_{\lambda max}$ values of 637.6–795.4, which were 12–15 times of that of the saturated conventional red MPs solution (Table 1).

Previously, some MPs-aa, such as MPs-Glu, MPs-Asp, MPs-Lys, MPs-His, MPs-Ser, and MPs-Thr, were speculated be more polar/hydrophilic than the conventional red MPs based on the retention times in HPLC, retention factor (R_f) values in TLC, as well as predicted LogP values (Jung et al., 2003; Kim et al., 2006). This speculation was confirmed experimentally in this study (Table 1). Nevertheless, it was observed that MPs-Arg exhibited a limited solubility in water, despite showing earlier retention times in HPLC (Fig. S6), and lower predicted LogP values when compared to the conventional red MPs rubropunctamine and monascorubramine (Fig. S7). It has been reported that the solubility process of a substance is complex that the final solubility is influenced by both dissociation from the solid state and solvation of the molecule by the solvent (Wassvik, Holmen, Draheim, Artursson, & Bergstrom, 2008; Bergstrom & Larsson, 2018). It's possible that the actual solubility of MPs-Arg in water was restricted by their solid-state properties, including crystal structure and melting point (T_m).

3.4. Color appearance of MPs-aa under different pH

The visual color appearance of different MPs-aa was investigated from pH 3.0 to pH 11.0. In the pH range of 3.0–9.0, all the MPs-aa exhibited a coincident red appearance, with no obvious differences in intensity and hue (Fig. 3 and Fig. S8). When pH raised to 11.0, the color of MPs-aa changed from red to pale yellow, and the UV–Vis spectra revealed clear hypochromic shifts (Fig. 3 and Fig. S8), indicating that they were unstable at high alkaline pH.

In contrast, the conventional red MPs rubropunctamine and monascorubramine exhibited distinct pH-dependent behaviors. Under a neutral to alkaline condition with a pH range of 7.0–11.0, the conventional red MPs exhibited a typical red color, characterized by two absorption peaks at 358 nm and 496 nm; while under an acidic condition with a pH range of 3.0–5.0, the color changed into purple-red, characterized by two absorption peaks at 408 nm and 522 nm (Fig. 3). Shi, Chen, Pistolozzi, Xia, & Wu (2016) also reported the sensitiveness of the conventional red MPs rubropunctamine and monascorubramine to the change of pH, and found a similar blue shift ($\Delta\lambda$ = 28 nm) and pale shade when pH value increased from 4 to 6. Given that most foods are naturally acidic, with a pH range of 3.5 to 7.0, MPs-aa were superior to the conventional red MPs in terms of color stability when used as red food colorants.

3.5. Color degradation of MPs-aa upon thermal treatment

The phenomenon of color degradation is frequently observed in natural pigments, making it a major concern in the field of food coloring. The degradation of the colorant would result in the gradual fading of the respective color until complete decolorization occurs, resulting in food quality being compromised and food's shelf life being lessened.

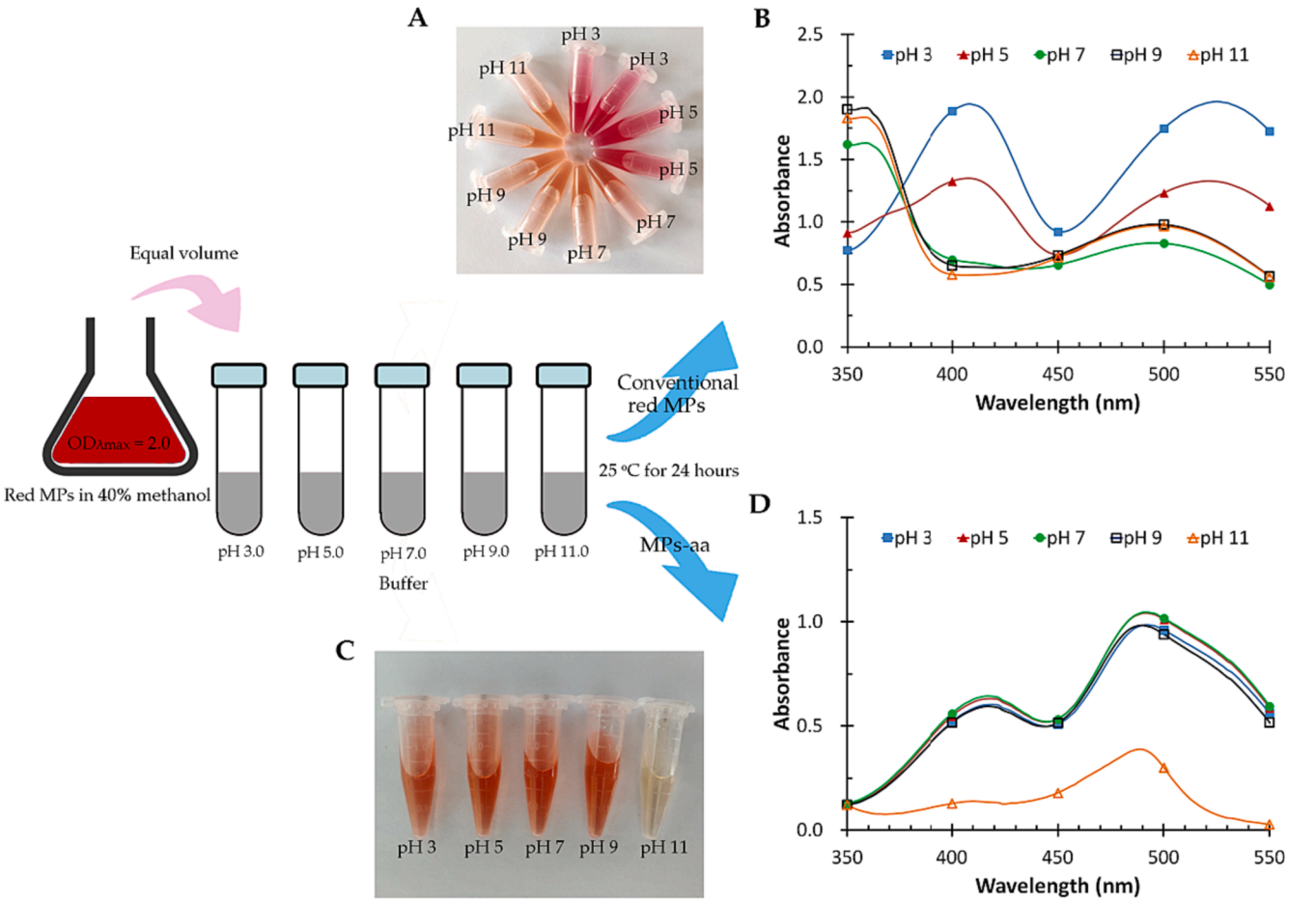

Fig. 3. Color appearance of MPs-aa under different pH. (A) and (B) the conventional red MPs rubropunctamine and monascorubramine; (C) and (D) the MPs-aa which were represented by MPs-Phe.

Thermal treatment is an essential process in the field of food preservation. Nevertheless, it has been observed that excessive heating led to considerable color degradation of the MPs produced by *M. ruber* in submerged fermentation (Vendruscolo, Müller, Moritz, de Oliveira, Schmidell, & Ninow, 2013). As shown in Fig. 4, in the pH range of 3.0–9.0, all the MPs-aa exhibited significantly better thermostability when compared to the conventional red MPs rubropunctamine and monascorubramine. The color degradation rate of MPs-aa was pH-dependent, and higher pigment stability was observed under-neutral to alkaline conditions (Fig. 4).

In the pH range of 3.0–7.0 (the pH range of most foods), MPs-His, which carry an amino acid residue with an imidazole ring, were the most stable one (Fig. 4). After being heated at 80 °C for 6 h at pH 3.0, 5.0 and 7.0, the remaining MPs-His was 2.6, 12.8 and 5.1 times greater than that of the conventional red MPs, respectively (Fig. 4). Following MPs-His, the MPs-aa showing good thermostability in pH range of 3.0–7.0 were those carrying aromatic amino acid residues, namely MPs-Phe, MPs-Tyr and MPs-Trp.

Thermal degradation of MPs-His, MPs-Phe, MPs-Tyr and MPs-Trp was further investigated at 65 °C, 75 °C, 85 °C and 95 °C using the model beverage system at pH 5.0. It was observed that the thermal degradation of these MPs-aa and the commercial anthocyanins-based food colorant GSE (the control) clearly followed the first-order reaction kinetic model with high regression coefficients ($0.9852 < R^2 < 0.9995$) (Fig. S9). This result was in agreement with previous studies that defined the first-order reaction model for the degradation of MPs (Vendruscolo et al., 2013) and anthocyanins (Cisse, Vaillant, Acosta, Dhuique-Mayer, & Dornier, 2009; Loypimai, Moongngarm, & Chottanom, 2016).

The kinetic rate constant (k) is an indicator that enables the prediction of the thermal degradation of pigments. A decrease in the k value corresponds to an improvement in the stability of the pigment. The k values of MPs-His, MPs-Phe, MPs-Tyr, MPs-Trp and GSE significantly increased ($p < 0.05$) as the temperature increased from 65 °C to 95 °C (Table 2), indicating these pigments degraded more rapidly at higher temperatures. At a consistent temperature, MPs-His exhibited the lowest k values and the greatest $t_{1/2}$ values, followed by MPs-Tyr, MPs-Trp and MPs-Phe; whereas GSE exhibited the greatest k values and the lowest $t_{1/2}$ values (Table 2). This observation suggested that MPs-His were more stable than MPs-Tyr, MPs-Trp and MPs-Phe, and all the four selected MPs-aa were more stable than the control GSE in the model beverage system upon thermal treatment. As thermal degradation of anthocyanins is a major problem for the food industry (Cisse et al., 2009), the four selected MPs-aa were promising alternatives for the natural red colorant GSE, with MPs-His being the preferred option. After classic low-temperature pasteurization (65 °C for 30 min), high temperature short time pasteurization (75 °C for 20 min, 85 °C for 15 min) and flash pasteurization (95 °C for 5 min), the estimated losses of MPs-His in the model beverage system were the lowest, about 1.02 %, 1.28 %, 3.31 % and 2.43 %, respectively (Table S2).

Currently, only a few MPs-aa, including MPs-Gly and MPs-Glu, have been subjected to assessments for their thermal stability (Wong & Koehler, 1983). Here, the thermal stability of 18 types of MPs-aa was investigated, and MPs-His, MPs-Tyr, MPs-Trp and MPs-Phe were firstly demonstrated to be the most thermally stable candidates, showing great potential for application in liquid food formulations. Furthermore, MPs-

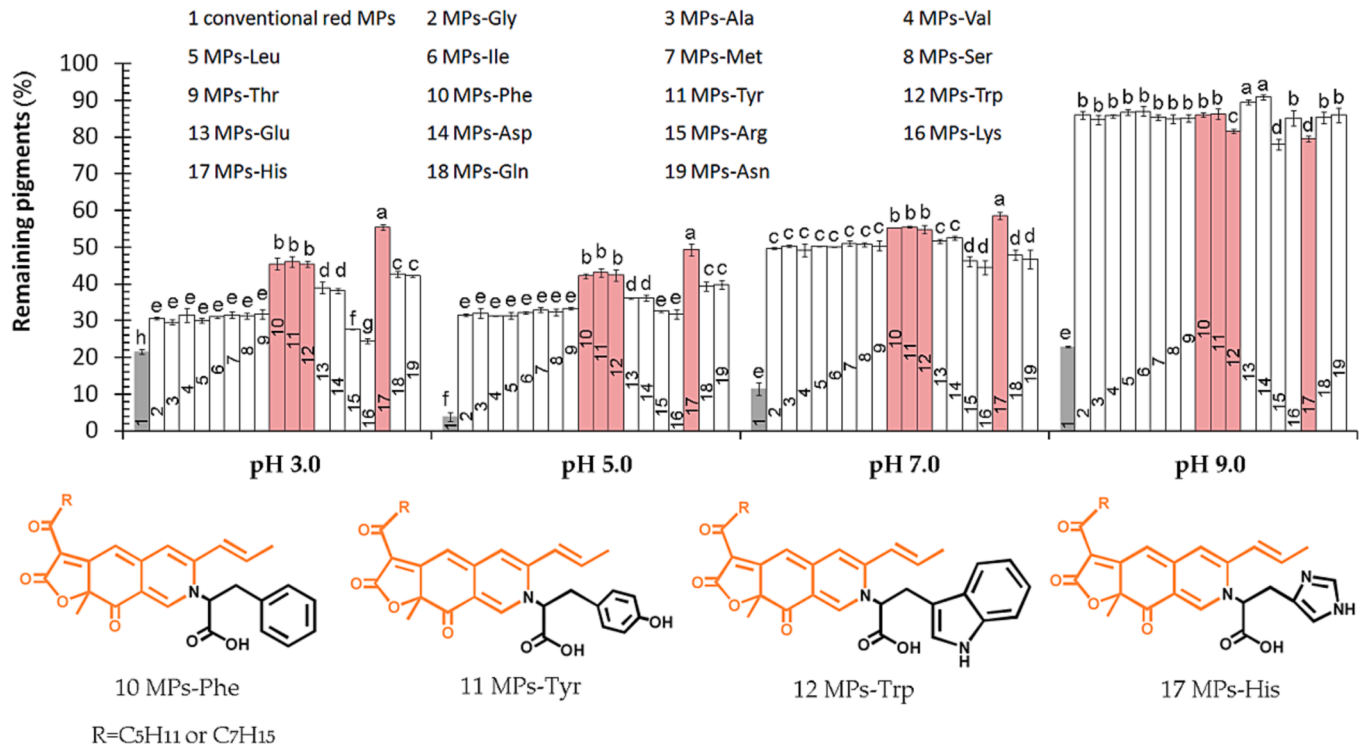

Fig. 4. The remaining pigments of different MPs-aa after being heated at 80 °C for 6 h under different pH conditions in a 20 % methanolic solution. The conventional red MPs rubropunctamine and monascorubramine were used as the control.

Table 2
Degradation kinetic parameters of MPs-His, MPs-Phe, MPs-Tyr and MPs-Trp in the model beverage system at pH 5.0 under different temperatures.

Pigments	$k \times 10^{-3}$ (min^{-1})				$t_{1/2}$ (min)			
	65 °C	75 °C	85 °C	95 °C	65 °C	75 °C	85 °C	95 °C
MPs-His	0.322 ± 0.016^{k}	0.648 ± 0.034^{j}	2.163 ± 0.126^{h}	4.785 ± 0.211^{g}	2150.7 ± 122.6^{a}	1069.8 ± 43.6^{b}	319.9 ± 15.8^{d}	144.7 ± 9.3^{e}
MPs-Tyr	1.831 ± 0.122^{i}	7.966 ± 0.424^{e}	11.842 ± 0.803^{d}	18.12 ± 1.014^{c}	380.7 ± 21.6^{c}	85.0 ± 6.3^{g}	58.9 ± 3.1^{h}	39.0 ± 2.0^{j}
MPs-Trp	1.683 ± 0.112^{i}	7.963 ± 0.326^{e}	11.331 ± 0.927^{d}	18.022 ± 1.016^{c}	413.6 ± 13.9^{c}	87.6 ± 3.3^{g}	60.8 ± 2.1^{h}	39.2 ± 2.0^{j}
MPs-Phe	1.692 ± 0.106^{i}	7.500 ± 0.504^{e}	12.709 ± 1.006^{d}	19.555 ± 1.032^{c}	410.4 ± 18.4^{c}	91.0 ± 4.0^{g}	55.7 ± 4.0^{h}	35.9 ± 2.1^{j}
GSE	5.352 ± 0.234^{f}	12.944 ± 0.931^{d}	24.01 ± 1.021^{b}	66.478 ± 2.016^{a}	129.7 ± 5.87^{f}	52.9 ± 2.1^{i}	30.3 ± 2.0^{k}	10.4 ± 0.4^{l}

Data are presented as mean ± standard deviation (n = 3). The commercial anthocyanins-based food colorant GSE was used as the control. Within the same parameter, values with different lower case letters indicate significant differences ($p < 0.05$).

Tyr, MPs-Trp and MPs-Phe have been found to exhibit antiobesity and/or antibacterial activities (Kim et al., 2006; Kim et al., 2010; Nam et al., 2014), which can bring the food product with additional advantages. Nevertheless, it has been noticed that the stability of some natural colorants can also be influenced by various additional factors, including oxygen, light, metal ions, antioxidants and so on (Náthia-Neves & Meireles, 2018; Møller et al., 2019). Therefore, further investigations are needed before using these red MPs-aa in industrial applications. Firstly, their stability should be assessed throughout all stages of the food production chain, including processing, packaging, storage, distribution and marketing, using different actual aqueous food systems. Secondly, stabilization techniques, such as copigmentation, encapsulation, and complexion (Albuquerque, Oliveira, Barros & Ferreira, 2020), should be employed for protection against their degradation.

4. Conclusions

A novel approach for the preparation of highly pure precursors of red MPs, namely monascorubrin and rubropunctatin, was developed. The novel approach is characterized by the two-stage, low-pH fermentation strategy and the downstream purification step of fractional crystallization, resulting an orangish red crystalline powder containing 36.0 % monascorubrin and 55.9 % rubropunctatin (totally 91.9 % precursors). The yield was about 0.79 g per 1 L of fermentation medium. Based on the most recent knowledge, this is the simplest, most convenient, and least expensive approach for the preparation of highly pure rubropunctatin and monascorubrin, which are indispensable precursors for the semi-synthesis of red MPs. The adequate preparation of pure monascorubrin and rubropunctatin is crucial in enabling the efficient production of red MPs by semi-synthesis on a large scale, hence facilitating extensive investigation into their properties and potential applications.

Via the azaphilic addition reaction of rubropunctatin and monascorubin, a total of 18 types of red MPs congeners carrying different amino acid moieties (MPs-aa) were semi-synthesized *in vitro*. The water solubility, pH and thermal stability of the MPs-aa were investigated. Although most of the MPs-aa exhibited similar characteristics in the improved water solubility and in the enhanced pH stability, they varied greatly in the aspect of thermal stability. MPs-His, MPs-Phe, MPs-Tyr and MPs-Trp were discovered to be among the most thermally stable group. The high thermal stability of MPs-His, MPs-Phe, MPs-Tyr, and MPs-Trp has never been reported before.

MPs-His, MPs-Phe, MPs-Tyr and MPs-Trp were then applied in the liquid food formulation of beverage. Their thermal degradation in the model beverage system at pH 5.0 was found to follow the first-order

reaction kinetic model. During the process of pasteurization, the estimated losses of MPs-His, MPs-Phe, MPs-Tyr and MPs-Trp were significant lower than that of the commercial anthocyanins-based natural food colorant, suggesting that the selected MPs-aa are promising alternatives for the anthocyanins-based red colorants. The findings in the present study provide water-soluble red MPs candidates with high stability, as well as a novel, attractive approach for their large scale production. These results would lead to new MPs products with improved quality and expanded application areas. However, further investigations are required for the practical application of these MPs-aa, including the synergistic effects of heat, oxygen, light, acidity, metal ions, antioxidants and different actual food components on their stability, as well as protection effects by the stabilization techniques such as copigmentation, encapsulation and complexion against their degradation.

CRediT authorship contribution statement

Linman He: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Cai Liu:** Methodology, Investigation, Writing – original draft. **Suo Chen:** Methodology, Investigation, Formal analysis. **Jialan Zhang:** Writing – review & editing. **Mengxiang Gao:** Supervision, Writing – review & editing. **Li Li:** Supervision, Formal analysis, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100940.

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