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Research Article

# Purification of polar compounds from *Radix isatidis* using conventional C18 column coupled with polar-copolymerized C18 column

Regarding hydrophilic interaction chromatography and normal phase liquid chromatography, RPLC is another choice used to separate polar compounds with the improvement of polar-modified C18 stationary phase. In this study, a method using conventional C18 column coupled with polar-copolymerized C18 column was successfully developed for the separation and purification of polar compounds from *Radix isatidis*, which is one of the most commonly used traditional Chinese medicines (TCMs). An XTerra MS C18 column was used to fractionate the extract of *R. isatidis* and a homemade polar-copolymerized C18 column was utilized for the final purification due to its good separation selectivity and high resolution for polar compounds. The established purification system demonstrated good orthogonality for the polar compounds. As a result, ten compounds were purified and three of them were identified as 3-methyl-5-vinyloxazolidin-2-one (compound **A**), 5-hydroxymethyl-2-furaldehyde (compound **B**) and 3-methylfuran-2-carboxylic acid (compound **G**) based on the MS, IR and extensive NMR data, respectively. It was demonstrated to be a feasible and powerful technique for the purification of polar compounds under RPLC mode and more chemical information of TCMs will be obtained to interpret the efficiency of TCMs.

**Keywords:** Banlangen / Polar compounds / Polar-copolymerized C18 / *Radix isatidis* / RP/RP-LC
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## 1 Introduction

Recently, traditional Chinese medicines (TCMs) are attracting more and more attention for their potential bioactivities. As one of the most widely used TCMs, *Radix isatidis* (Banlangen in Chinese) was proved to have a variety of pharmacological activities [1–4]. Therefore, it is necessary to study the chemical constituents of *R. isatidis* for bioactive validation. Several methods have been developed for the separation and purification of medium and weak polar compounds from *R. isatidis*, including flash chromatography [5], high-speed counter-current chromatography [6] and HPLC [7]. However, no reports have focused on the polar compounds in *R. isatidis*, which are expected, since water is used as the traditional extraction solvent for the preparation of most TCMs.

RPLC is the most popular technique for the separation of medium and weak polar compounds. However, the separation of polar compounds on RPLC was difficult due to strong hydrophobicity of C18. As a traditional method used for the separation of polar compounds, normal phase liquid chromatography is limited in the preparative separation of polar compounds for the inadequate solvating power. Also, hydrophilic interaction chromatography (HILIC) usually has the poor separation resolution especially in preparative scale. With the improvement of stationary phase, polar-modified C18 was developed and enhanced the ability for the separation of polar compounds with 100% aqueous compatibility, which demonstrated different selectivity compared to conventional C18 [8–11]. Xia *et al.* [12] reported that Atlantis dC18 was successfully used for the separation and purification of pyrodoxal, folic acid and caffeine. Therefore, it is hopeful to separate the polar compounds from natural products by using polar-modified C18.

As far as we know, hundreds or even thousands of compounds are contained in TCMs. Based on this fact,

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**Abbreviations: HILIC,** hydrophilic interaction chromatography**; TCMs,** traditional Chinese medicines

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these complicated samples should be fractionated first. And then, these fractions are purified on another column with different selectivity. In the process of compounds purification, several chromatographic separation modes can be selected and different systems can be constructed for the separation of complex matrix with different properties, in which RPLC coupled with reversed phase liquid chromatography (RP/RP-LC) was one of the most prospective separation system owing to its high separation efficiency. The orthogonality of the RP/RP-LC system was limited for the separation of medium and weak polar compounds. However, it will be good for the separation of polar compounds by using the separation system consisting of conventional C18 and polar-modified C18 [13, 14]. Recently, a new type of polar-modified RPLC stationary phase was developed and named polar-copolymerized C18 in our lab, which had good selectivity and better resolution ability especially for the polar compounds separation [15].

In this study, the potential of purification of polar compounds under RP mode from complex matrix was explored. A further objective of this study was to purify kinds of polar compounds from natural products and biological samples by using the developed method in this work.

## 2 Materials and methods

### 2.1 Apparatus and reagents

An Agilent 1100 (USA) HPLC system was used to optimize the chromatographic conditions in analytical experiments, including a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment and a diode array detector. Data acquisition and processing were conducted by Agilent ChemStation software. A Waters Auto-Purification Factory was utilized for the fractionation, consisting of a 2777 sample manager, a passive splitter, a 515 pump as the compensate pump, an MUX-UV 2488 two-channel UV detector, a Micromass ZQ2000 two-channel MS detector, two 2525 binary gradient modules and a 2757 sample manager. Waters Masslynx workstation was used as the data processing software. A Waters Alliance HPLC system was adopted for the final purification, consisting of a 2695 HPLC pump and 2996 photodiode array detection. Data acquisition and processing were conducted by Waters Empower software. A Waters Fraction Collector III was used to collect fractions in the final purification. NMR experiments were run on a Bruker Avance II 400 spectrophotometer and $D_2O$ was used as the solvent.

The columns used in this study were listed as follows: homemade 5 μm C18 HC (made in-house, according to the report [15] with minor modification: without endcapping; the polar-copolymerized stationary phase composed of mixed *n*-octadecyl and chloropropyl (C18-C3Cl) was synthesized by using horizontal polymerization technique which reduces the activity of silanols and enhances the stability of the bond phases [15]; the carbon content of this stationary phase is 11.59%), Atlantis 5 μm T3 C18 (Waters), TSKgel 3 μm ODS-100V C18 (Tosoh), Spursil EP 5 μm C18 (Dikma), XBridge 3.5 μm C18 (Waters), Zorbax Eclipse 5 μm XDB C18 (Waters), Inspire 5 μm C18 (Dikma), XTerra 5 μm MS C18 (Waters). All the columns were 150 mm × 4.6 mm format except XTerra 5 μm MS C18 (150 mm × 2.1 mm). The columns used for the purification in this work were XTerra MS C18 (150 mm × 50 mm, 5 μm, Waters) and C18 HC (150 mm × 10 mm, 5 μm, made in-house).

HPLC-grade acetonitrile was purchased from TEDIA (Fairfield, USA). Formic acid and trifluoroacetic acid (HPLC grade) were purchased from Acros (Cambridge, USA). Methanol was purchased from YuWang (ShanDong, China). Water was prepared by a Milli-Q system (Millipore, Billerica, MA, USA). Industry-grade acetonitrile was only used for the fractionation and was purchased from JinMa (ShanDong, China).

### 2.2 Chromatographic conditions

In fractionation procedure, the mobile phases were A1: 0.2% formic acid aqueous solution, and B1: acetonitrile. Gradient elution steps were as follows: 0–25 min, 5% → 25% B1; 25–35 min, 25% → 100% B1; 35–45 min, 100% → 100% B1. The time of equilibrium was set at 15 min. The monitoring wavelength was 280 nm. Flow rate was set at 100 mL/min and the injection volume was 5 mL. The ratio of passive splitter was 1:4000, the flow rate of the compensating solvent was 1.0 mL/min, and the compensating solvent was acetonitrile–water–formic acid (40:60:0.2, v/v/v).

For the final purification, the mobile phases were A2: 0.1% trifluoroacetic acid aqueous solution, and B2: acetonitrile containing 0.1% trifluoroacetic acid. Gradient elution steps were as follows: 0–60 min, 0% → 5% B2; 60–80 min, 5% → 5% B2. The monitoring wavelength was 260 nm. Flow rate was set at 4 mL/min and injection volume was 100 μL.

### 2.3 Sample preparation

*R. isatidis* was collected from NingXia province and authenticated by Mr. Xiaoping Yang, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. Twenty kilogram of the herb was decocted in 200 L of *n*-butanol at 120°C for 120 min. After filtration, the residue was collected and redecocted in 200 L of *n*-butanol at 120°C for 90 min. The decoctions were combined and dried by rotary evaporation at 60°C in vacuum. Then, 560 g of extract was dissolved in 8 L of methanol and mixed with 600 g silica gel (100–200 mesh). The mixture was brought onto a Buchner funnel after the solvent evaporated thoroughly. And then, it was eluted by 7.5 L of *n*-hexane and subsequently 15 L of

methanol. Finally, the methanol eluted fraction was dried by rotary evaporation in vacuum at 40°C. About 120 g methanol eluted fraction was dissolved in 0.5 L of methanol at a concentration of 240 mg/mL and filtered through 0.22 μm membranes for further isolation.

## 3 Results and discussion

### 3.1 Fractionation of the extract of *R. isatidis*

It is a well-known fact that polar constituents are weakly retained on conventional reversed phase columns in comparison with the medium and weak polar constituents. Based on this property, conventional C18 (XTerra MS C18) was adopted as the preparative column for the fractionation of the polar constituents with the format of 150 mm × 50 mm. Preparative columns with large inner diameter can provide enough sample loading for the further purification and structural identification. In order to obtain satisfactory separation profiles, optimization of the chromatographic conditions is important before the preparative process. A method used to transfer the operation conditions from analytical scale to preparative scale was developed in our previous work [16]. Using the same method as mentioned above, the chromatographic conditions for the fractionation were optimized on an XTerra MS C18 column with the inner diameter of 2.1 mm (150 mm × 2.1 mm, 5 μm, Waters), which was packed with the same stationary phase as the XTerra MS C18 column with the inner diameter of 50 mm (150 mm × 50 mm, 5 μm, Waters). And then, about 120 g extract of *R. isatidis* was fractionated in 50 injections and 39 fractions (Fr.1–Fr.39) were collected at 1 min intervals from 1 to 40 min automatically. As shown in Fig. 1, reproducibility of different injections is acceptable. Satisfactory reproducibility in preparative chromatography is required for effective simplification of complex matrix and continuous purification of target compounds. Fractions eluted in the same retention time in different injections were combined and dried by rotary evaporation at 40°C in vacuum. As a result, extract of *R. isatidis* was fractionated into 39 fractions according to the retention time of the eluted compounds.

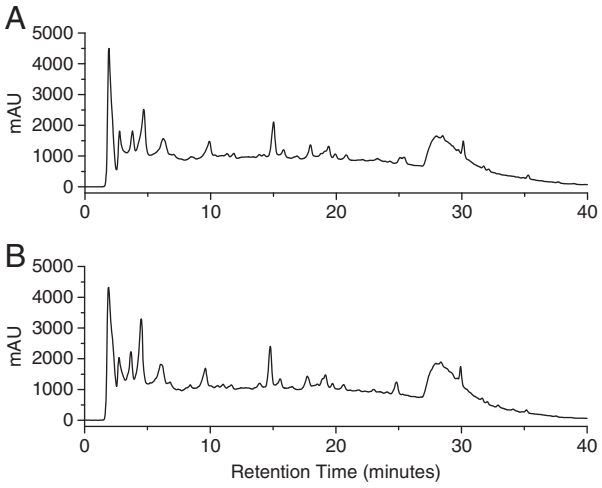



**Figure 1.** Fractionation of *R. isatidis* on XTerra MS C18 column (150 mm × 50 mm) in different injections, (A) the 6th injection and (B) the 48th injection. Conditions: mobile phase A1, 0.2% formic acid aqueous solution and B1, acetonitrile; gradient: 0–25 min, 5%→25% B1; 25–35 min, 25%→100% B1; 35–45 min, 100%→100% B1; injection volume: 5 mL; flow rate: 100 mL/min; UV detection: 280 nm.

### 3.2 Evaluation of columns for the separation of polar compounds

HILIC is a suitable technique for the separation of polar compounds, but few publications reported the purification of polar compounds under HILIC mode due to its poor separation resolution. With the development of polar-modified C18, RPLC was employed for the purification of polar compounds in the present work.

In the procedure of fractionation, extract of *R. isatidis* was fractionated according to the polarity of constituents. Fractions eluted between 1 and 9 min (Fr.2–Fr.9) were studied for their relatively strong polarity. For example, fraction 7 (Fr.7) was selected in random for the comparison of the separation capability between commercial columns and homemade C18 HC. The commercial columns consisted of the conventional C18 (XBridge 3.5 μm C18, Zorbax Eclipse 5 μm XDB C18 and Inspire 5 μm C18) and polar-modified C18 (Atlantis 5 μm T3 C18, TSKgel 3 μm ODS-100V C18 and Spursil EP 5 μm C18). As shown in Fig. 2, C18 HC (Fig. 2A) and commercial polar-modified C18 (Fig. 2B, C and D) have higher resolution and better selectivity than the conventional C18 (Fig. 2E, F and G). Retentions of polar compounds were enhanced significantly by introducing polar groups which increased the polar interaction between the solutes and absorbents. C18 HC (Fig. 2A) and Spursil EP C18 (Fig. 2D) show the best selectivity for Fr.7 compared to other commercial polar-modified C18. But, selectivity between C18 HC and Spursil EP C18 was quite different and the former had the longer retention for the polar compounds. Atlantis T3 C18 (Fig. 2B) and TSKgel ODS-100V C18 (Fig. 2C) can retain the polar compounds well, but several peaks combined together for the lower separation resolution. In order to avoid the de-wetting effect, 5% B2 isocratic elution was employed on the conventional C18 columns, but the retention of polar compounds was still poor. Based on the discussion mentioned above, separation of the polar constituent Fr.7 on homemade C18 HC shows better resolution and retention. And thus, separation of other polar constituents (Fr.2–Fr.6 and Fr.8–Fr.9) on the homemade C18 HC column were tested. As shown in Fig. 3, separation of these constituents on this column shows good separation resolution. Fraction 2 (shown in Fig. 3A), which is the most polar constituent eluted from 1–2 min, is also well separated on C18 HC

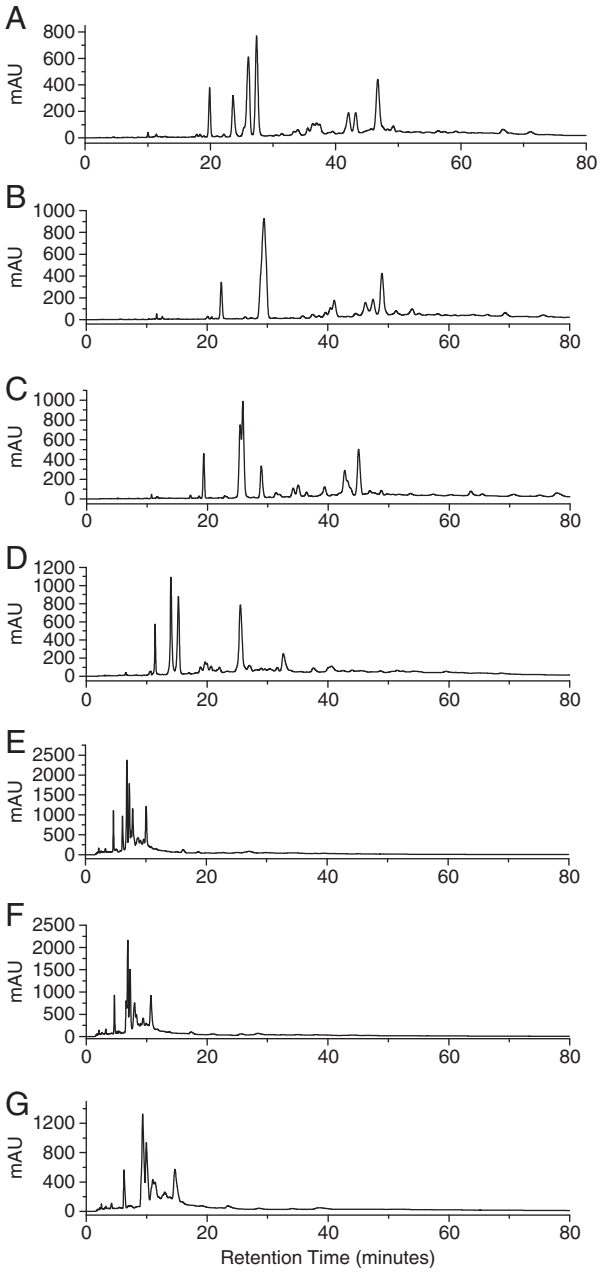



**Figure 2.** Separation of Fr.7 on (A) homemade polar-copolymerized C18 (C18 HC) column (150 mm × 4.6 mm) and commercial columns ((B) Atlantis 5 μm T3 C18; (C) TSKgel 3 μm ODS-100V C18; (D) Spursil EP 5 μm C18; (E) XBridge 3.5 μm C18; (F) Zorbax Eclipse 5 μm XDB C18; (G) Inspire 5 μm C18); conditions: mobile phase A2: 0.1% trifluoroacetic acid aqueous solution, and B2: acetonitrile containing 0.1% trifluoroacetic acid; gradient for A–D: 0–60 min, 0%→5% B2; 60–80 min, 5%→5% B2 and gradient for E–G: 0–80 min, 5%→5%; formats of these columns were all the same: 150 mm × 4.6 mm; the monitoring wavelength: 280 nm; flow rate: 0.85 mL/min and injection volume is 3 μL; column temperature: 30°C.

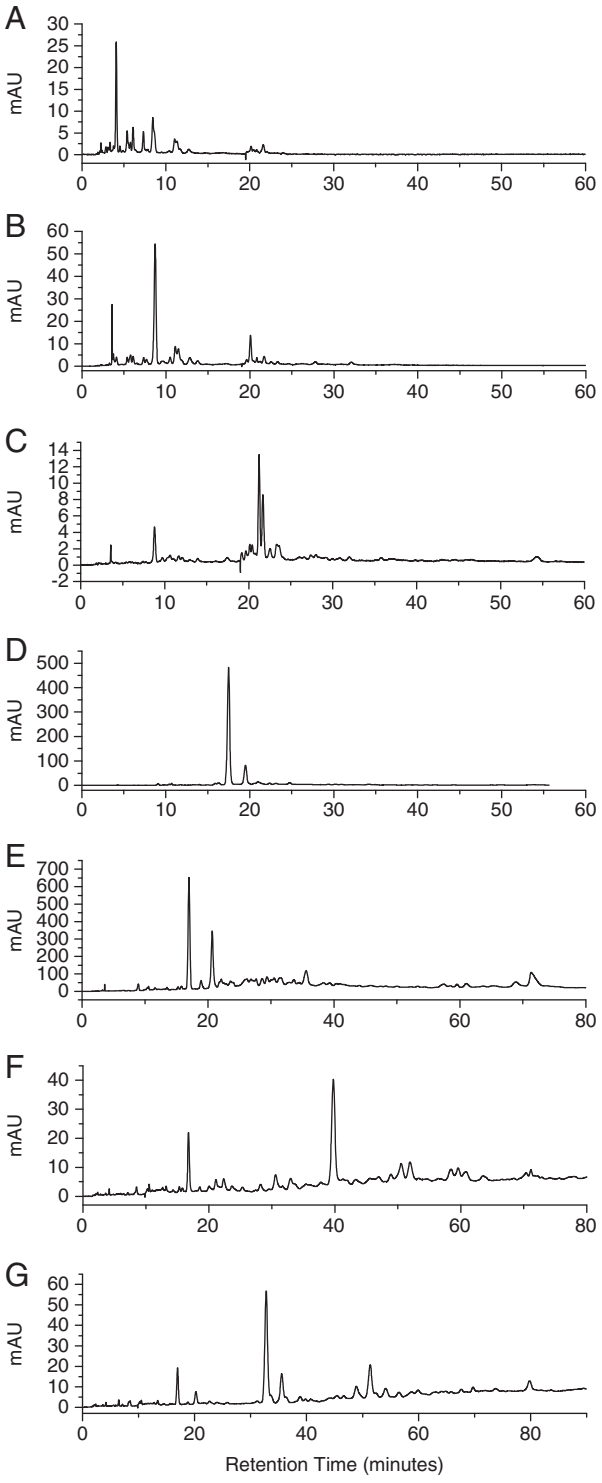



**Figure 3.** Separation of the polar fractions except Fr.7 (A. Fr.2; B. Fr.3; C. Fr.4; D. Fr.5; E. Fr.6; F. Fr.8; G. Fr.9) on C18 HC column (150 mm × 4.6 mm). Conditions: mobile phase A2: 0.1% trifluoroacetic acid aqueous solution, and B2: 0.1% trifluoroacetic acid acetonitrile; gradient for A, B, C: 0–10 min, 0%→0% B2; 10–60 min, 0%→5% B2; gradient for D, E, F: 0–60 min, 0%→5% B2; 60–80 min, 5%→5% B2; gradient for G, H: 0–60 min, 0%→5% B2; 60–90 min, 5%→10% B2; the monitoring wavelength: 280 nm; flow rate: 0.85 mL/min and injection volume is 10 μL; column temperature: 30°C.

column. In an overview of the separation profiles of these polar constituents, they were well separated on the C18 HC due to the different selectivity between XTerra MS C18 column and C18 HC column. So, the well-designed C18 HC was employed for the separation and purification of polar constituents.

### 3.3 Purification of Fr.7

The combined system consisting of the XTerra MS C18 column and C18 HC column was established and used for the separation of polar constituent Fr.7. Optimization of chromatographic conditions was the same to the procedures mentioned above [16]. After optimizing chromatographic factors carefully, it was found that trifluoroacetic acid used as an additive can get higher separation resolution than formic acid. So, trifluoroacetic acid was utilized as the additive and chromatographic conditions were stated in Section 2.2. As shown in Fig. 4, about 1.5 g of Fr.7 is isolated in 110 injections. Fractions were collected by Waters Fraction Collector III at 0.3 min intervals automatically and the fractions were combined according to the retention time of *per* compound. ''Heart-cutting'' was employed as the repeated isolation and combination strategy to insure the purity of these compounds. At last, the combined fractions were dried by rotary evaporation under 40°C in vacuum for NMR analysis. As demonstrated in Fig. 4, ten compounds (named compounds **A–J**) were purified with the satisfactory purity (purity >90%). It is noteworthy that the recoveries of compounds **D** and **G** were sacrificed to ensure their higher purity.

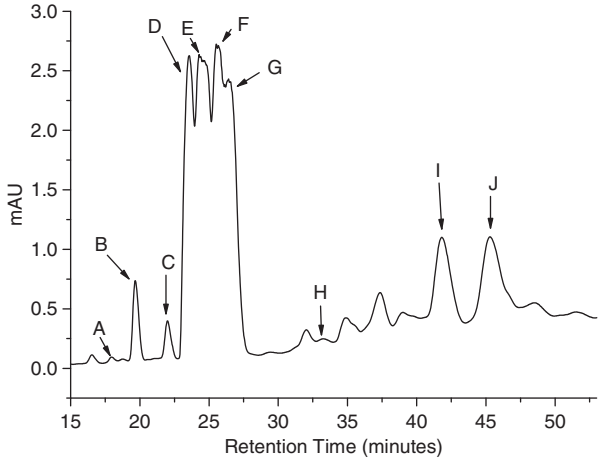



**Figure 4.** Purification of Fr.7 on C18 HC column (150 mm × 10 mm). Conditions: mobile phase A2: 0.1% trifluoroacetic acid aqueous solution, and B2: 0.1% trifluoroacetic acid acetonitrile; gradient: 0–60 min, 0%→5% B2; 60–80 min, 5%→5% B2; the monitoring wavelength: 260 nm; flow rate: 4 mL/min and injection volume is 100 μL; column temperature: 30°C.

Obviously, purified sample amount can be greatly improved by increasing the injection volume and inner diameter of the C18 HC column. In this work, we are trying to develop the method for the separation and purification of polar compounds from TCMs. Large injection volume and big inner diameter column will be employed to improve the purification efficiency in the future work. It is prospective that many new compounds will be purified from other polar constituents.

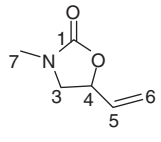
### 3.4 Identification of compounds

As demonstrated in Section 3.3, ten compounds were purified with high purity. Among them, three were characterized by MS, IR and NMR. Identification of other compounds is ongoing in our laboratory. These three compounds (shown in Fig. 5) are identified as 3-methyl-5-vinyloxazolidin-2-one (compound **A**), 5-hydroxymethyl-2-furaldehyde (compound **B**) and 3-methylfuran-2-carboxylic acid (compound **G**), respectively. Compound **A** [17] was isolated for the first time as natural compound and compound **G** [18–22] as first found in *R. isatidis*.

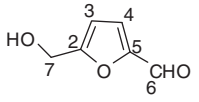
Identifications of these compounds were listed as follows:

Compound **A**, yellow solid; positive HR-ESI-MS *m/z:* 128.0736 ($[M+H]^+$, calculated for $C_6H_{10}NO_2$, 128.0711); IR $\nu_{max}$ ($CH_3OH$) $cm^{-1}$: 1677, 1449, 1204, 1141; $^1$H-NMR (400 MHz, $D_2O$): δ 5.90 (1H, ddd, $J$ = 17.2, 10.4, 6.8 Hz, H-5), 5.35 (1H, d, $J$ = 17.2 Hz, H-6), 5.27 (1H, d, $J$ = 10.4 Hz, H-6′), 5.10 (1H, dt, $J$ = 6.8, 8.8 Hz, H-4), 3.72 (1H, dd, $J$ = 8.8, 8.8 Hz, H-3), 3.31 (1H, dd, $J$ = 9.2, 6.8 Hz, H-3′), 2.62 (3H, s, -$CH_3$); $^{13}$C-NMR (100 MHz, $D_2O$): δ 161.8 (C-1), 134.2 (C-5), 119.1 (C-6), 78.3 (C-4), 45.6 (C-3), 38.7 (C-7).

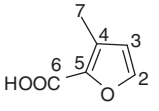
Compound **B**, brown solid; positive HR-ESI-MS *m/z:* 127.0384 ($[M+H]^+$, calculated for $C_6H_7O_3$, 127.0395); IR $\nu_{max}$ ($CH_3OH$) $cm^{-1}$: 3339, 1677, 1207, 1142; $^1$H-NMR (400 MHz, $D_2O$): δ 9.58 (1H, s, H-6), 7.66 (1H, d, $J$ = 3.6 Hz, H-4), 6.80 (1H, d, $J$ = 3.6 Hz, H-3), 4.82 (2H, s, H-7, H-7′); $^{13}$C-NMR (100 MHz, $D_2O$): δ 180.5 (C-6), 161.3 (C-2), 151.8




compound **A**
3-methyl-5-vinyloxazolidin-2-one




compound **B**
5-hydroxymethyl-2-furaldehyde




compound **G**
3-methylfuran-2-carboxylic acid

**Figure 5.** Structures of compound **A**, compound **B** and compound **G**.

(C-5), 126.8 (C-4), 111.0 (C-3), 56.0 (C-7). The $^{1}$H- and $^{13}$C-NMR spectral data were in agreement with those of 5-hydroxymethyl-2-furaldehyde [23].

Compound **G**, brown solid; positive HR-ESI-MS *m/z:* 127.0381 ($[M+H]^+$, calculated for $C_6H_7O_3$, 127.0395); IR $\nu_{max}$ ($CH_3OH$) $cm^{-1}$: 3256, 1683, 1617, 1203, 1141; $^{1}$H-NMR (400 MHz, $D_2O$): δ 7.95 (1H, d, $J = 4$ Hz, H-2), 6.458 (1H, d, $J = 4$ Hz, H-3), 2.32 (3H, s, -$CH_3$); $^{13}$C-NMR (100 MHz, $D_2O$): δ 175.0 (C-6), 156.0 (C-2), 154.7 (C-5), 141.8 (C-4), 113.3 (C-3), 14.0 (C-7). The $^{1}$H-NMR spectral data were in agreement with those of 3-methylfuran-2-carboxylic acid [24].

## 4 Concluding remarks

A practical RP/RP-LC system was developed for the purification of polar compounds from *R. isatidis* using the conventional XTerra MS C18 column and homemade polar-copolymerized C18 column as the fractionation and final purification column respectively. Polar constituents of extract of *R. isatidis* were fractionated based on the retention time of compounds on the conventional C18 column. Fr.7 was selected as an example for further purification for the method validation. It showed that the RP/RP-LC system had good separation power for the polar constituent. As a result, Fr.7 was well separated on the homemade C18 HC column compared to other commercial columns. Totally, ten compounds were purified and three of them were identified by MS, IR and NMR. Compound **A** was found to be 3-methyl-5-vinyloxazolidin-2-one, which was isolated for the first time from natural products. Compounds **B** and **G** were determined as 5-hydroxymethyl-2-furaldehyde and 3-methylfuran-2-carboxylic acid, respectively. Compound **G** was separated for the first time from *R. isatidis*. As an alternative method used to separate polar compounds under RPLC mode, the established method was demonstrated to be feasible and potent for the separation and purification of polar compounds from natural products. Based on this approach, more and more information about polar compounds in TCMs would be obtained to explain the pharmacological effects of TCMs to some extent.

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*The authors have declared no conflict of interest.*

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