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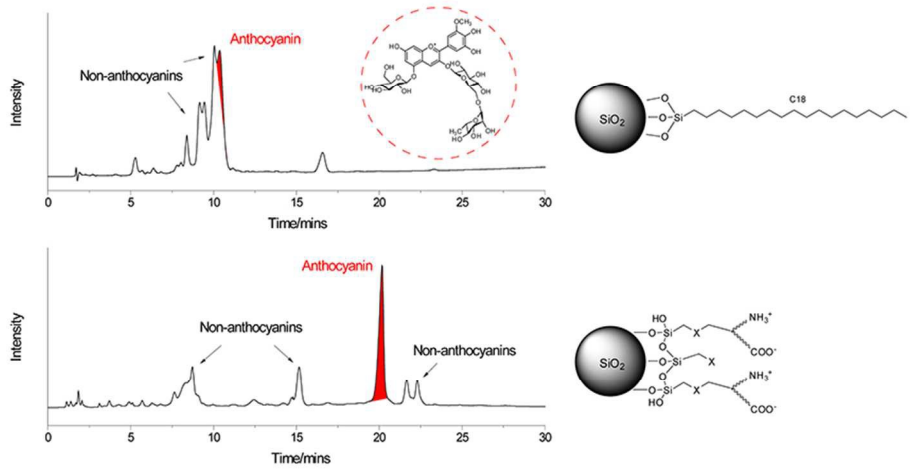

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Preparative separation of a challenging anthocyanin from *Lycium ruthenicum* Murr. by two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography

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Abstract

The preparative separation of anthocyanins by HPLC often suffers from insufficient separation selectivity. In this work, a two-dimensional liquid (LC-LC) method was established to efficiently purify a challenging anthocyanin in *Lycium ruthenicum* Murray. Reversed phase liquid chromatography (RPLC) was used in the first-dimension preparation to fractionate the sample for its high separation efficiency. After the optimization of second-dimension methods, hydrophilic interaction chromatography (HILIC) was applied to further isolate the anthocyanin for the good orthogonality to RPLC. To improve HILIC separation for anthocyanins, stationary phases and mobile phases were investigated systematically. A satisfactory result was obtained on a zwitterionic Click XIon column with 1% phosphoric acid as acidic additives. Using the above method, the anthocyanin and three new alkaloids were isolated from *L. ruthenicum* for the first time. This RPLC/HILIC method solved the coelution problem of the anthocyanin and basic non-anthocyanins in one-dimensional HPLC, benefiting from the significantly improved separation resolution.

Introduction

Anthocyanins, a class of flavonoid subgroups, are water-soluble pigments responsible for various colors in most fruits and plants. As an important natural pigment, anthocyanins are added to foods as an alternative to synthesized colorants proven to increase risk of diseases to humans in chronic consumption [1]. In addition, there is mounting evidence to demonstrate that anthocyanins possess a number of potential health benefits [2-4]. Nonetheless, most bioactive investigations on anthocyanins are studied with crude extracts of plants due to the high cost of anthocyanin standards, leading an encertain evaluation of anthocyanins. Hence, preparation of pure anthocyanins from natural plants is desired for their bioactivity researches.

High performance liquid chromatography (HPLC), owing to its good reproducibility and separation efficiency, has been perceived as one of the most important techniques for purification of anthocyanins [5-7]. However, at present, anthocyanin separation is almost exclusively performed in reversed phase liquid phase (RPLC) with conventional C18 columns [8]. This often results in insufficient separation selectivity. To deal with this issue, the common method is to use low pressure column chromatography coupled with RPLC [9]. The separation efficiency and velocity would decrease with this method. Alternatively, a promising solution is the development of novel HPLC methods for anthocyanin purification. In recent years, mixed-mode chromatography (MMC) has been used in the analysis and purification of anthocyanins [10-12]. In our previous work [12], a MMC method was established to purify anthocyanins from natural plant, based on a mixed-mode reversed phase/strong anion-exchange column. This method exhibited improved separation selectivity toward anthocyanins, especially for *cis-trans* isomers. Nevertheless, the applicable scope of the mixed-mode purification is limited. HPLC methods with complementary selectivity are of great importance for anthocyanin preparation.

Hydrophilic interaction chromatography (HILIC) [13] has attracted increasing attention for its special ability to separate polar compounds [14-16]. De Villiers et al. [17] have firstly utilized this mode for analysis of anthocyanins. Unique chromatographic

behaviors of anthocyanins are observed, ascribed to the distinct separation mechanisms. Unfortunately, several drawbacks, such as poor sample solubility and unsatisfactory peak shape, have hampered the application of HILIC in the purification of anthocyanins. Further investigation is necessary.

Two-dimensional liquid chromatography (LC-LC) provides a powerful capability to separate compounds from complex samples, because of significant improvement in separation selectivity [18-21]. Recently, De Villiers et al. [22] established a comprehensive 2D-HILIC/RPLC method for analysis of anthocyanins. Improved separation was obtained for the combination of various retention mechanisms. The results indicated the potential capability of LC-LC in anthocyanin separation. Nonetheless, preparation of anthocyanins with this technique is rarely reported.

Lycium ruthenicum Murray, belonging to the family Solanaceae, is a fruit mainly growing in the northwest part of P. R. China. It has been widely used to produce beverages for a long time, because it is tasty,. In addition, *L. ruthenicum* is also used as a traditional medicine to treat diseases, such as abnormal menstruation and menopause. Many researchers have reported that *L. ruthenicum* possess abundant anthocyanins [23, 24]. To date, few anthocyanins have been separated from this plant for structure identification. In our previous work, six anthocyanins in *L. ruthenicum* were isolated and identified [12]. However, one type of anthocyanin in this plant has not been purified for structure analysis, due to the co-elution with many basic non-anthocyanins in one-dimensional RPLC. The subject of this study was to develop a RPLC/HILIC method for efficient purification of this challenging anthocyanin from *L. ruthenicum*.

Materials and Methods

Reagents and materials

The fruits of *Lycium ruthenicum* were hand-picked in Dulan (Qinghai, China) (latitude, 36° 26′ N; longitude, 96° 31′ E; altitude, 2774 m). Acetonitrile (ACN) was purchased from Merck of HPLC grade (Darmstadt, Germany) and from Yuwang Chemical Reagent Factory of industrial grade (Shandong, China). Methanol was purchased from Yuwang Chemical Reagent Factory of HPLC grade. Trifluoroacetic

acid (TFA) and formic acid (FA) were purchased from J&K chemical of HPLC grade (Hebei, China). Phosphoric acid was purchased from Tedia of HPLC grade (Fairfield, USA). Water for the HPLC mobile phase was reverse osmosis Milli-Q water (18.2 MΩ, Millipore, Billerica, MA, USA). The reference malvidin 3-*O*-[6-*O*-(4-*O*-(*cis*-*p*-coumaroyl)-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside]-5-*O*-[*β*-D-glucopyranoside] (A1), petunidin 3-*O*-[6-*O*-(4-*O*-(*trans*-*p*-coumaroyl)-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside]-5-*O*-[*β*-D-glucopyranoside] (A2) and petunidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-*trans*-(*β*-D-glucopyranoside)-*p*-coumaroyl)-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside]-5-*O*-[*β*-D-glucopyranoside] (A3) were isolated in our laboratory and identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR). Their structures were shown in Fig. 1.

The columns used were listed as follow: XTerra MS C18 (4.6×150 mm, 5 μm, Waters, Milford, MA, USA), XCharge C8SAX (4.6×150 mm, 5 μm, Acchrom, Beijing, China), XCharge C18 (4.6×150 mm, 5 μm, Acchrom, Beijing, China), Atlantis HILIC Silica (4.6×150 mm, 5 μm, Waters, Milford, MA, USA), XAmide (4.6×150 mm, 5 μm, Acchrom, Beijing, China), Click TE-GSH synthesized in our lab [25], and Click XIon (4.6×150 mm, 5 μm, Acchrom, Beijing, China). The representative surface chemistry of Click XIon column was shown in Fig. 2.

Instrumentation

Preparative separation was performed on the Purification Factory (Waters, Milford, MA, USA). This LC system consists of two 2525 binary gradient modules (Waters, Milford, MA, USA), autosampler (Leap Technologies, Carrboro, NC, USA), a 2498 ultraviolet (UV) detector (Waters) and MassLynx software (Waters, version 4.1).

Chromatographic analysis was carried out on an Alliance HPLC system consisting of a Waters 2695 HPLC pump and a 2489 UV–vis detector. Data acquisition and processing were conducted by Waters Empower software (Milford, MA, USA).

MS was performed on a Q-TOF Premier (Waters MS Technologies, Manchester,

U.K.). NMR spectra were measured in CD_3OD/TFA-*d* (95:5, v/v) solution and recorded on a Bruker DRX-400 spectrometer (Rheinstetten, Germany), using TMS as an internal standard. Chemical shifts were reported in units of δ (ppm) and coupling constants (*J*) were expressed in Hz.

Extraction and pretreatment

The procedures were the same as our previous work [12]. The fruit of *Lycium ruthenicum* Murr. was extracted triply with 20 fold of 70% ethanol for 2 h at pH 2.5 (adjusted with hydrochloric acid). Subsequently, three filtrates were combined and concentrated by rotary evaporation at 50 °C in vacuum. Then, the aqueous extract was prepared on the AB-8 macroporous resin (50×520 mm, The Chemical Plant of NanKai University, Tianjing, China). The strong polar constituents were removed with aqueous acid (0.5% formic acid, v/v). Then target constituent was eluted with 70% ethanol. It was concentrated by rotary evaporation at 50 °C in vacuum, and finally was lyophilized.

The anthocyanin sample was dissolved in aqueous acid, and was subjected to an strong cation-exchange solid phase extraction cartridge (40 mL, 20 g sorbent, Acchrom), preconditioned successively with MeOH and distilled water (0.5% formic acid). Non-anthocyanin compositions were collected with 3 vol of 5% acetonitrile (0.5% formic acid). Subsequently, anthocyanins were eluted with 3 vol of 30% acetonitrile (1 M NaH_2PO_4, pH 2.0). The anthocyanin solution was dried by rotary evaporation at 50 °C in vacuum to remove organic solvent as much as possible, and then loaded on the AB-8 macroporous resin. Phosphate was washed out by distilled water (0.5% formic acid). Anthocyanins were eluted with 70% ethanol. The anthocyanin sample was concentrated by rotary evaporation at 50 °C in vacuum.

The two dimensional liquid chromatography separation

The first-dimension preparation of anthocyanin constituents

The first-dimension preparation of the anthocyanin sample was carried out on a prep XTerra MS C18 column (50×150 mm, 5 μm Waters, Milford, MA, USA). The mobile phase A1 was 0.2% v/v TFA in water, and mobile phase B1 was 0.2% v/v TFA in methanol. Gradient elution steps were as follows: 0-40 min, 18~50% B1. The time

of equilibrium took 15 min. The flow-rate was 80 mL/min. Chromatograms were recorded at 520 nm. The target fraction was collected according to UV absorption intensity.

The second-dimension preparation of the target fraction

180 mg of the target fraction was dissolved in 10 mL of methanol and filtered through 0.22 μm pore size membranes to obtain the sample with concentration at about 18 mg/mL.

The second-dimension preparation was performed on a prep Click XIon HILIC column (20×250 mm, 10 μm, Acchrom, Beijing, China). The mobile phase A2 was 1% v/v phosphoric acid in water, and mobile phase B2 was 1% v/v phosphoric acid in ACN. Gradient elution steps were as follow: 0-30 min, 10~35% A. The flow rate was 19 mL/min. Chromatogram was recorded at 280 nm. Three fractions (F1-1, F1-2, and F1-3) were obtained. To remove the phosphoric acid in the sample, these fractions were further separated on an XCharge C18 column (20 × 250 mm, 10 μm, Acchrom, Beijing, China). The mobile phase A3 was 5% v/v FA in water, and mobile phase B3 was ACN. The same isocratic elution condition, which was 6% B3, was used for the three fractions. Chromatograms for F1-1 and F1-3 were recorded at 280 nm, and that for F1-2 was at 520 nm.

The chromatographic conditions

The analytical HPLC experiments were performed at the flow rate of 1.0 mL/min. Column temperature was maintained at 30 °C throughout using a thermostat. Detection was carried out at 520 nm and 280 nm, unless otherwise specified.

HPLC analysis of fractions and pure compounds were conducted on an XTerra MS C18 column (4.6×150 mm, 5 μm). The mobile phase A was 0.2% TFA (v/v) in water, and B was 0.2% TFA (v/v) in methanol. Gradient elution steps were as follows: 0-30 min, 15%~50% B; 30-40 min, 90% B.

The separation of the target fraction in MMC was performed on an XCharge C8SAX (4.6×150 mm, 5 μm) and XCharge C18 (4.6×150 mm, 5 μm) columns, respectively. The mobile phase A was 5% FA (v/v) in water, and B was 5% FA (v/v) in ACN. Gradient elution steps were as follows: 0~30 min, 1%~7% B on an XCharge

C8SAX column, and 0~30 min, 3%~15% B on an XCharge C18 column.

For column selection of HILIC method, the mobile phase A was 5% FA (v/v) in water, and B was 5% FA (v/v) in ACN. Gradient elution steps were as follows: 0~30 min, 10%~40% A.

The mobile phase optimization of HILIC method was carried out on Click XIon (4.6×150 mm, 5 μm) column with 5% FA, 0.2% TFA and 1% phosphoric acid as acidic additives. The other conditions were the same as above section.

The separation of the target fraction in HILIC was performed on a Click XIon column (4.6×150 mm, 5 μm). The mobile phase A was 1% phosphoric acid (v/v) in water, and B was 1% phosphoric acid (v/v) in ACN. Gradient elution steps were as follows: 0~30 min, 10%~32% A.

Results and discussion

The first-dimension preparation

Prior to HPLC separation, pretreatment procedures were used to remove non-anthocyanins, such as sugars and phenolic compounds etc., from the extracts. The crude extracts of *L. ruthenicum* were pretreated by AB-8 macroporous resin to eliminate strong and weak polar compositions in the extracts. The anthocyanin sample was eluted with 70% v/v ethanol. Subsequently, based on the positive charge nature of anthocyanins in acidic condition, strong cation-exchange (SCX) solid phase extraction (SPE) was adopted to separate anthocyanin-rich constituent. Its anthocyanin profile was shown in Fig. 3, and seven anthocyanins were marked as P1-P7.

P2, P3, P4, P5, P6, and P7 had been isolated in our previous work [12]. In this work, the purification of P1 would be fully investigated. The first-dimension preparation was performed on a prep XTerra MS C18 column to fractionate the sample for its high separation efficiency. The target fraction was collected according to UV absorption intensity (shown in supporting information). The HPLC analysis result was shown in Fig. 4. Using the XTerra MS C18 column, P1 was effectively separated from the other anthocyanins in *L. ruthenicum* according to the hydrophobicity (Fig. 4A). The complexity of the sample was significantly reduced. However, as can be seen in Fig. 4B, many non-anthocyanins still co-eluted with the anthocyanin on the conventional

C18 column. Therefore, one-dimensional RPLC separation was unavailable to efficiently purify the anthocyanin, related to the limited separation selectivity.

The optimization of the second-dimension preparation

The attempt of the mixed-mode purification

MMC are able to bring different separation selectivity to RPLC for the involvement of complex separation mechanisms. In this section, two mixed-mode reversed phase/strong anion-exchange stationary phases with different bonding groups were evaluated to separate the target fraction. The XCharge C8SAX column was used for its different separation selectivity toward anthocyanins [12]. And the XCharge C18 column was tested for its superior ability to separate basic compounds [26, 27]. Unfortunately, poor resolution between P1 and non-anthocyanins was observed on the both columns (Fig. 5). According to the preceding preparation procedures (SCX SPE and RPLC separation), it is possible that the target anthocyanin in the fraction had similar charge characteristic and hydrophobicity to the non-anthocyanins. And thus, mixed-mode reversed phase/strong anion-exchange stationary phases failed to provide adequate separation resolution for these two types of compounds. The mixed-mode columns were inappropriate as the second-dimension preparation.

The development of HILIC method for anthocyanin separation

HILIC can offer alternative separation selectivity to RPLC, because of the distinct retention mechanisms [21]. Reportedly, the retention of anthocyanins in HILIC mode mainly depended on the hydrophilic glycosyls [17]. Hence, this chromatographic mode might be usable to solve the coelution problem in the first-dimension separation. However, HILIC methods for anthocyanin preparation are rarely reported. A proper one should be developed.

Selection of HILIC columns

Choosing stationary phases with proper retention to analytes is one of the most important parameters in HPLC method development. In this work, four HILIC columns with different bonded groups were evaluated using three anthocyanins (A1, A2, and A3) (Fig. 1) under the identical conditions. Atlantis HILIC Silica column consists of bare silica phase. XAmide column contains amide groups bonded to the

silica surface. Click TE-GSH column possesses glutathione attached to the surface, and Click XIon column contains zwitterionic groups covalently grafted to the silica (Fig. 2). The separation results were presented in Fig. 6. Very weak retentions for the anthocyanins were observed on the Atlantis HILIC Silica (Fig. 6A) and the XAmide columns (Fig. 6B). The anthocyanins were eluted nearly in dead time. On the Click TE-GSH column (Fig. 6C), retentions were enhanced. In contrast, Click XIon column exhibited strongest retentions for the anthocyanins. Good retention not only benefited the optimization of separation, but alleviated the poor solubility problem in HILIC. The Click XIon column was selected in this work.

The mobile phase optimization

To obtain satisfactory peak shape and resolution, different acidic additives, including 5% FA, 0.2% TFA, and 1% phosphoric acid, were investigated (Fig. 7). Adding 5% FA in the mobile phase (Fig. 7A), broad peaks for three anthocyanins were observed. By contrast, anthocyanin peak shapes were improved significantly with 0.2% TFA (Fig. 7B). This is mainly ascribed to its strong acidity. Nonetheless, it was noticed that there was a significant decrease in retention for three anthocyanins presented. TFA is a kind of ion-pairing agent with great ion pair abilities [28], which would diminish the hydrophilicity of anthocyanins. Thus, the retentions were reduced, which was detrimental to the preparative separation. When 1% phosphoric acid was used (Fig. 7C), sharp peak shapes and good retention for the anthocyanins were achieved. Baseline resolution between A1 and A2 appeared. To summarize, based on the Click XIon column, a HILIC method was developed to separate anthocyanins using acetonitrile/water (1% phosphoric acid) as mobile phases.

The second-dimension preparation of HILIC

The target fraction was separated using the optimized HILIC method (Fig. 8). The compounds, which co-eluted in RPLC and MMC (Fig. 4 and 5), dispersed across the chromatogram. This result demonstrated the good orthogonality of this HILIC method to RPLC. Hence, the second-dimension preparation of the target fraction was carried out on a prep Click XIon column (shown in the supporting information). Three fractions (F1-1, F1-2 and F1-3) were collected according to UV intensity.

Subsequently, an XCharge C18 column was used to purify the fractions to remove phosphoric acid in the fractions and yield high purity compounds (shown in the supporting information). Eventually, P1 was isolated along with three non-anthocyanin compounds (F1-1-1, F1-1-2 and F1-3-1). The analysis results demonstrated that the HPLC purity of these four compounds were more than 98% (Fig. 9). Interestingly, it was noticed that they had almost identical retention times on the RP column, indicating the considerable difficulty of purification of P1 with one-dimensional RPLC. However, after the second-dimension preparation of HILIC, P1 was easily collected with favorable purity. Thus, this RPLC/HILIC method allowed the purification of compounds co-eluting in one-dimensional RPLC, ascribed to the improved separation resolution.

The structure elucidation

Four compounds were isolated in this work, including an anthocyanin and three non-anthocyanins. The identification of these four compounds was listed as follows:

P1 was obtained as red powder. $[M+H]^+$: *m/z* 787.2296, calculated for $C_{34}H_{43}O_{21}$, *m/z* 787.2291.The ^1H NMR data was presented in Table 1. By comparing the ^1H NMR and NOESY data with the literature [5], P1 was identified as petunidin-3-*O*-[6-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside].

F1-1-1 was yielded as white powder. $[M+H]^+$: *m/z* 636.3115, calculated for $C_{31}H_{45}N_3O_{11}$, *m/z* 636.6127. The ^1H and ^{13}C NMR spectra were presented in Table 2. The ^1H and ^{13}C NMR data were very similar to those of N^1,N^{10}-didihydrocaffeoylspermidine [29], except for a set of additional signals arising from a glucose moiety. In the ^1H NMR spectrum, the signal of the anomeric proton presented at δ 4.75 (d, J = 7.5 Hz), and the assigned glucose protons possessed the coupling constants J = 7.0-11.0 Hz, indicating the glucose residue of F1-1-1 was in the β-D-glucopyranose form. The attachment of the sugar unit at the position of 7'-OH was determined by NOESY correlation of H-1''' (δ_H 4.75 d, J = 7.5 Hz) of the glucose with the H-8' (δ_H 7.09 d, J = 8.2 Hz) of the N^1, N^{10}-didihydrocaffeoylspermidine. F1-1-1 was identified as 7'-*O*-[β-D-glucopyranose]-N^1,

N^{10}-didihydrocaffeoylspermidine.

For the other two non-anthocyanins, tentative identification was performed based on MS, ^{1}H and ^{13}C NMR data. The 2D NMR results were not obtained, since these two compounds were easily degraded in organic solvent.

F1-1-2 was yielded as white powder. $[M+H]^+$: *m/z* 634.2954, calculated for $C_{31}H_{44}N_3O_{11}$, *m/z* 634.2970. The ^{1}H and ^{13}C NMR data was very similar to N^1-dihydrocaffeoyl-N^3-caffeoyspermidine [29], except for a set of additional signals arising from a glucose moiety. In the ^{1}H NMR spectrum (shown in the supporting information), the signal of the anomeric proton presented at δ 4.85 (d, J = 7.2 Hz), and the assigned glucose protons possessed the coupling constants J = 7.0-11.0 Hz, indicating the glucose residue of F1-1-2 was in the β-D-glucopyranose form. The attachment of the sugar unit was not determined. Compared to F1-1-1, F1-1-2 was tentatively identified as 7"-*O*-[β-D-glucopyranose]-N^1-dihydrocaffeoyl-N^3-caffeoyspermidine.

F1-3-1 was obtained as white powder. $[M+H]^+$: *m/z* 796.3486, calculated for $C_{37}H_{53}N_3O_{16}$, *m/z* 796.3499. The ^{1}H and ^{13}C NMR data was very similar to those of F1-1-2, except for one more β-D-glucopyranoses (δ_H 4.75 d, J = 7.5 Hz) presented (^{1}H NMR data shown in the supporting information). The attachment of the sugar units was not determined. Compared to F1-1-1, F1-3-1 was tentatively identified as 7'-*O*-[β-D-glucopyranose]-7"-*O*-[β-D-glucopyranose]-N^1-dihydrocaffeoyl-N^3-caffeoys permidine.

The chemical structures of the isolated compounds were presented in Fig. 10. To our best knowledge, all the compounds were separated from *L. ruthenicum* for the first time. F1-1-1, F1-1-2, and F1-3-1 were three new structurally related alkaloids, and reported in *L. ruthenicum* for the first time. The results not only confirmed the above deduction about the structural type of non-anthocyanins, but also warned us that the basic compounds in the plants would coelute with anthocyanins in SCX SPE process.

Conclusion

A RPLC/HILIC method was successfully developed for the preparative separation of a challenging anthocyanin in *L. ruthenicum*. RPLC was used in the first-dimension preparation to obtain the target fraction for the good separation efficiency. The anthocyanin was effectively separated from the other anthocyanins in *L. ruthenicum*, according to hydrophobicity. After the optimization of second-dimension methods, HILIC was applied to further isolate the anthocyanin, owing to the good orthogonality to RPLC. To improve separation of anthocyanins in HILIC, stationary phases and mobile phases were investigated systematically. The results showed that satisfactory separation could be achieved on a zwitterionic Click XIon column with 1% phosphoric acid as acidic additives. Based on the established method, four compounds, including one anthocyanin and three new alkaloids, were isolated from *L. ruthenicum*. These compounds were all separated from this plant for the very first time. All the results indicated that the RPLC/HILIC method was efficient in preparative separation of the anthocyanin from complex mixtures, as ascribed in the improved separation resolution. Moreover, this method can be a potent option for the purification of anthocyanins from other natural plants.

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Figure captions

Fig. 1. Chemical structures of the isolated anthocyanins from *L. ruthenicum*.

Fig. 2. Representative surface chemistry of the Click Xion stationary phase.

Fig. 3 HPLC chromatogram of the anthocyanin-rich constituent. Mobile phase A: 0.2% TFA (v/v) aqueous solution and B: 0.2% TFA (v/v) in methanol; gradient: 0~30 min, 15%~50% B; flow rate: 1 mL/min; Temperature: 30 °C; wavelength: 520 nm.

Fig. 4. HPLC chromatograms of the target fraction on the XTerra MS C18 (4.6×150 mm, 5 μm) at (A) 520 nm and (B) 280 nm. Mobile phase A: 0.2% TFA (v/v) aqueous solution and B: 0.2% TFA (v/v) in methanol; gradient: 0~30 min, 15%~50% B; Other conditions are the same as those in Fig. 3.

Fig. 5. HPLC chromatograms of the target fraction on the (A) XCharge C8SAX (4.6×150 mm, 5 μm) and (B) XCharge C18 (4.6×150 mm, 5 μm) columns. The mobile phase A: 5% FA (v/v) in water, and B: 5% FA (v/v) in ACN; gradient: 0~30 min, 1%~7% B on the XCharge C8SAX column, and 0~30 min, 3%~15%B on the XCharge C18 column; wavelength: 280 nm. Other conditions are the same as those in Fig. 3.

Fig. 6. HPLC chromatograms of the reference anthocyanins on (A) Atlantis HILIC Silica (4.6×150 mm, 5 μm), (B) XAmide (4.6×150 mm, 5 μm), (C) Click TE-GSH (4.6×150 mm, 5 μm), and (D) Click XIon (4.6×150 mm, 5 μm) columns. The mobile phase A: 5% FA (v/v) in water, and B: 5% FA (v/v) in ACN; gradients: 0~30 min, 10%~40% A; wavelength: 280 nm. Other conditions are the same as those in Fig. 3.

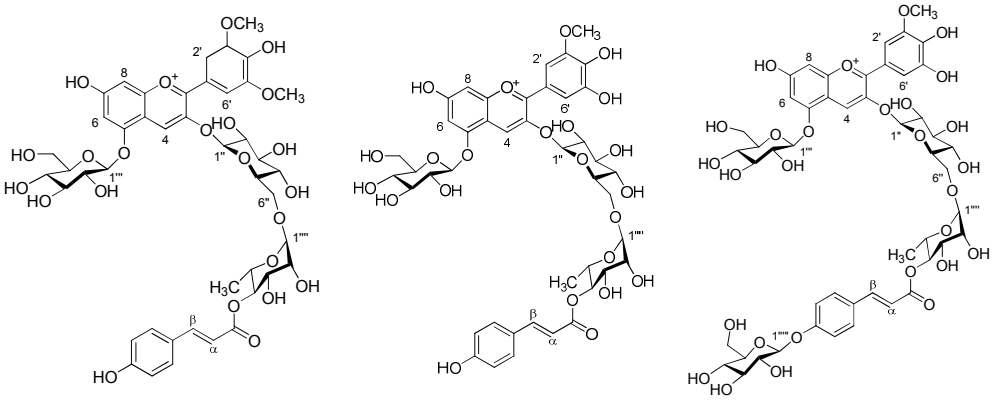
Fig. 7. HPLC chromatograms of the reference anthocyanins on the Click XIon column (4.6×150 mm, 5 μm) with (A) 5% FA, (B) 0.2% TFA, and (C) 1% phosphoric acid as acidic additives. Mobile phase A: different additives in waters, and B: those in ACN; gradients: 0~30 min, 10%~40% A; wavelength: 280 nm. Other conditions are the same as those in Fig. 3.

Fig. 8. HPLC chromatogram of the target fraction on the Click XIon column (4.6×150 mm, 5 μm). Mobile phase A: 1% phosphoric acid in water, and B: 1% phosphoric acid in ACN; gradients: 0~30 min, 10%~32% A; wavelength: 280 nm. Other conditions are the same as those in Fig. 3.

Fig. 9. HPLC purity evaluation of the prepared compounds on the XTerra MS C18 column (4.6×150 mm, 5 μm). F1-1-1 and F1-1-2 were isolated from the F1-1; P1 was isolated from the F1-2; F1-3-1 was isolated from F1-3; wavelength: 280 nm. Other

conditions are the same as those in Fig. 3.

Fig. 10. The chemical structures of the isolated compounds.



A1 A2 A3

Fig. 1

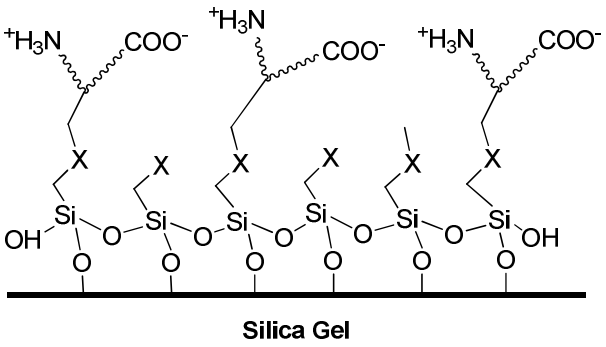


Fig. 2

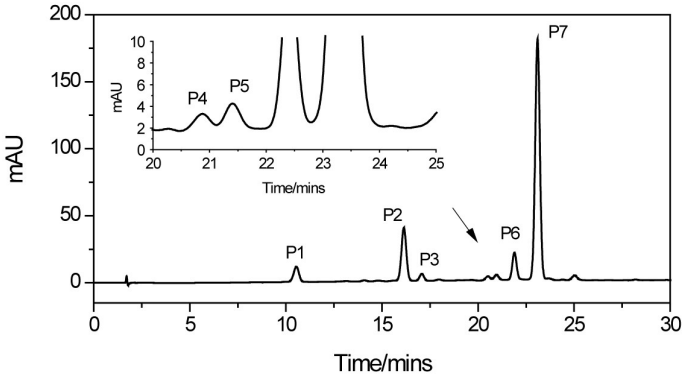


Fig. 3

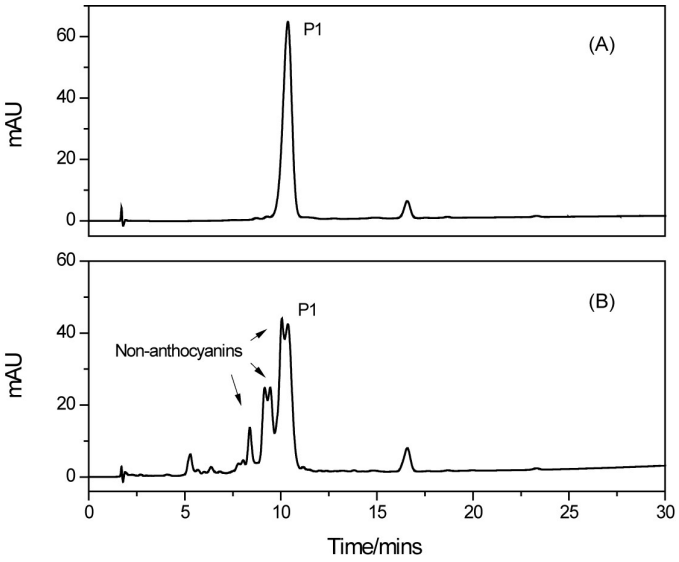


Fig. 4

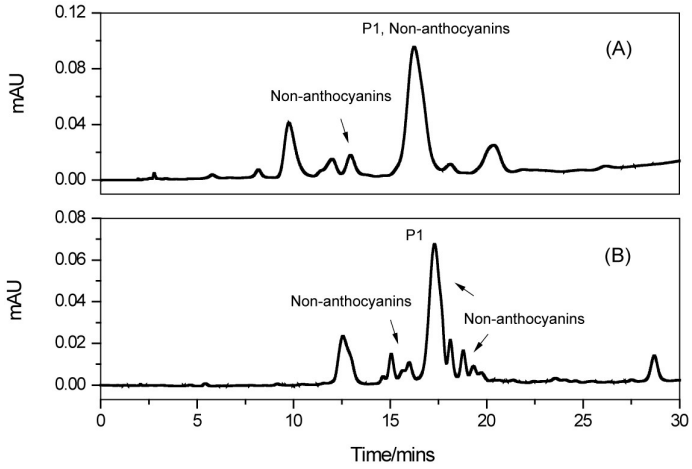


Fig. 5

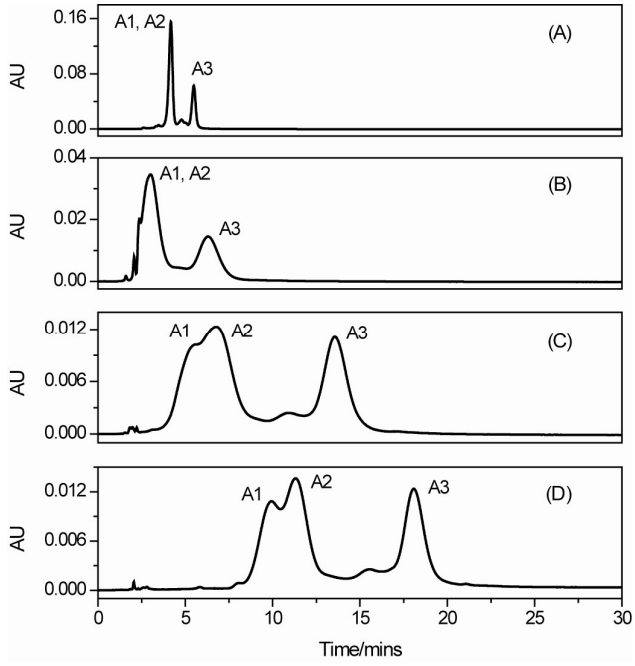


Fig. 6

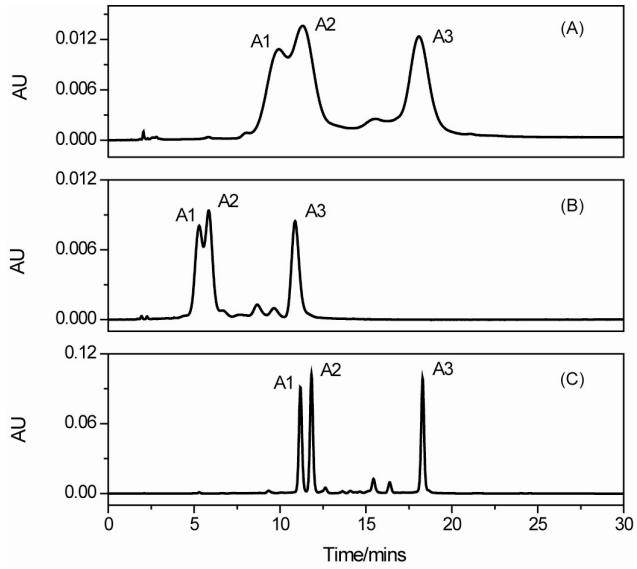


Fig. 7

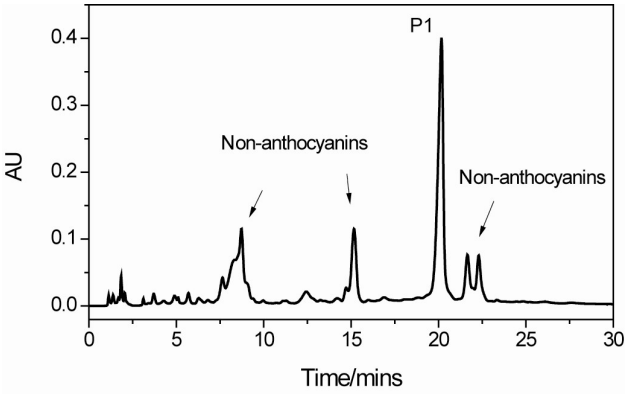


Fig. 8

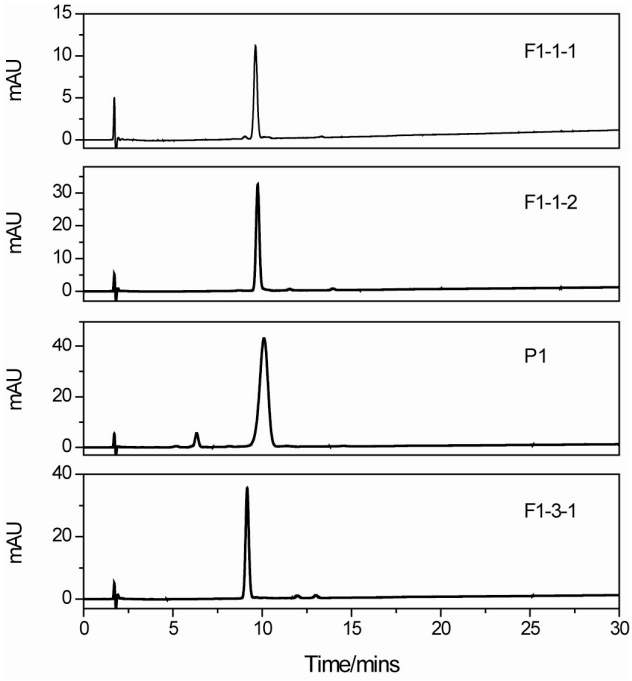


Fig. 9

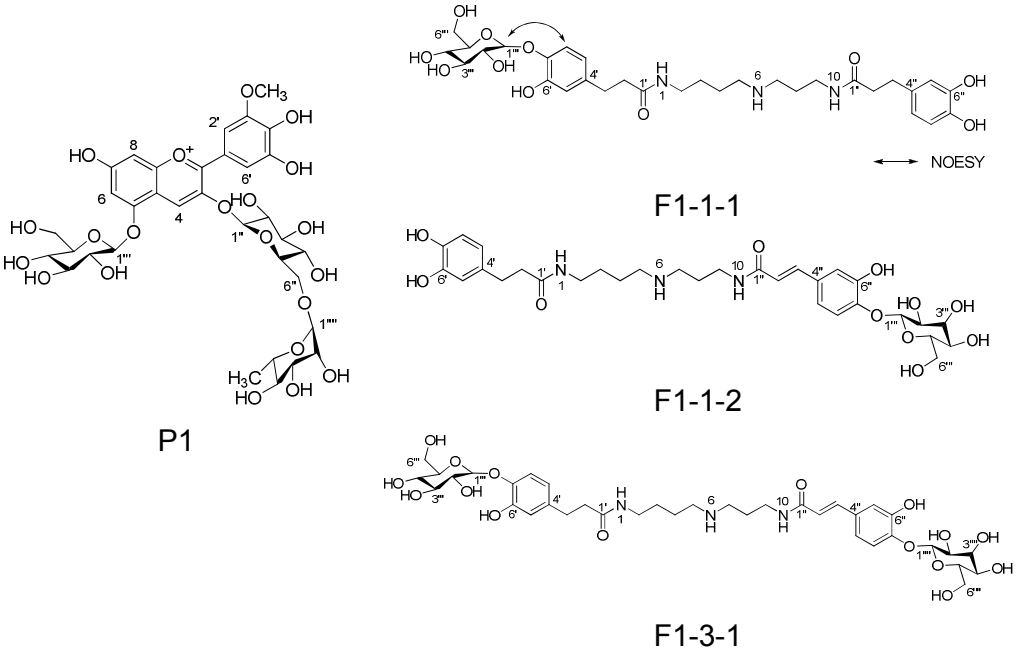


Fig. 10

Table 1 ^{1}H NMR data for the isolated anthocyanins in CD_3OD/TFA-*d* (95:5, v/v)

H	P1
Anthocyandin	
4-H	8.93 s
6-H	7.04 s
8-H	7.08 s
2'-H	7.82 s
5'-H	
6'-H	7.97 s
3'-OCH3	4.01 s
5'-OCH3	
3-*O*-Glucopyranoside	
1''	5.51 d (7.8)
2''	3.75–3.62
3''	
4''	
5''	
6a	3.98
6b	4.04
5-*O*-Glucopyranoside	
1'''	5.22 d (7.9)
2'''	3.87–3.60
3'''	
4'''	
5'''	
6a	
6b	3.88
6''-*O*-Rhamnopyranosyl	
1''''	4.66 s
2''''	3.60–3.28
3''''	
4''''	
5''''	
-CH_3	1.15 d (6.2)

Table 2 NMR data for the isolated alkaloids in CD_3OD/TFA-*d* (95:5, v/v)

	F1-1-1	
position	^{1}H	^{13}C
1		
2	3.18 t (5.6)	38.98
3	1.51 overlap	27.39
4	1.55 overlap	24.45
5	2.83 overlap	48.61
6		
7	2.67 t (7.0)	45.9
8	1.77 m	27.6
9	3.25 t (6.3)	36.56
10		
1’		175.46
2’	2.46 overlap	48.45
3’	2.85 overlap	32.34
4’		137.84
5’	6.74 d (1.9)	117.39
6’		148.16
7’		145.21
8’	7.09 d (8.2)	118.58
9’	6.74 d (1.9)	121.04
1’’		176.68
2’’	2.52 overlap	32.82
3’’	2.78 overlap	31.94
4’’		133.45
5’’	6.67 overlap	116.46
6’’		146.18
7’’		144.69
8’’	6.70 overlap	116.85
9’’	6.55 dd (8.0, 2.0)	120.78
7’-*O-Glucopyranoside*		
1’’’	4.75 d (7.5)	102.34
2’’’		73.4
3’’’	3.52-3.39	76.17
4’’’		69.92
5’’’		76.92
6’’’a	3.75 m	61.03
6’’’b	3.89 m	61.03