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Research Article

A novel two-dimensional preparative chromatography method designed for the separation of traditional animal Tibetan medicine Osteon Myospalacem Baileyi

Animal medicine is an important part in traditional Tibetan medicine. However, information about the chemical composition of animal medicine is very limited, and there is a lack of comprehensive chromatographic purification methods. In the present work, animal medicine Osteon Myospalacem Baileyi was taken as an example and a novel two-dimensional preparative chromatographic method was established for the preparation of single compounds with high purity from the extract of Osteon Myospalacem Baileyi. The first-dimension preparation was carried on a DAISO Silica prep column, and ten fractions were obtained from the 112.3 g crude sample within 12 injections. A diol prep column used in nonaqueous mobile phase was selected for the second-dimension preparation. The purity of the compounds isolated from the crude extract was >98%, which indicated that the method built in this work was efficient to manufacture single compounds of high purity from the extract of Osteon Myospalacem Baileyi. Additionally, this method showed great potential in the purification of weakly polar chemicals and it could act as a good example in the purification of other traditional animal medicines.

Keywords: Diol prep column / Osteon Myospalacem Baileyi / Silica prep column / Tibetan medicine / Two-dimensional separation
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1 Introduction

Osteon Myospalacem Baileyi, known as Sai long gu (Tibet language, means "blind rat bone"), is the whole skeleton of Tibet plateau rodentia animal *Myospalacem Baileyi*. Osteon Myospalacem Baileyi has been widely used in the Tibet region and since 1991 Osteon Myospalacem Baileyi has been listed in the Pharmacopoeia of People's Republic of China as a first-class animal new medical material. The biological activities of Osteon Myospalacem Baileyi crude extract have been proved by modern pharmacological research, including antirheumatoid arthritis [1], anti-osteoporosis [2], analgesia [3], and anti-inflammation [4]. However, the complex Osteon Myospalacem Baileyi extract makes the purification so difficult that very little information about the chemical composition has been reported with much less on the chemical compounds responsible for therapeutic effects. It is very desirable to establish a method for obtaining single compounds from Osteon Myospalacem Baileyi for the following pharmacological activity research.

In our previous work, separating chemicals from Osteon Myospalacem Baileyi mainly relied on traditional separation methods (unpublished data), such as column chromatography, TLC, and exclusion chromatography. However, because of successive extractions by solvents, successive column isolation, low column efficiency, bad separation repeatability, and poor column resolution, only some fractions with low pharmacology activity were obtained.

Preparative HPLC, one of the most efficient technologies applied in the preparation of single chemicals in complex samples, takes the advantages of high performance separation, online monitoring, and automatic control to realize the efficient preparation of single compounds [5]. Its application in separating compounds from complex nature products attracts more and more attention. However, taking the complex chemical composition of the nature medicine extract into account, we think it is impossible to obtain compounds of high purity by only 1D preparation due to the limited resolution and peak capacity.

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Two-dimensional HPLC has become a powerful tool to separate compounds from complex samples, since it has made a great improvement in separation selectivity and peak capacity [6]. Two-dimensional RP–RPLC is a practical separation method and had been applied for the isolation of compounds in *Lignum Dalbergiae Odoriferae* extracts [5]. Two-dimensional HILIC coupled with RP chromatography has been proved to be very useful in the separation of *Stevia rebaudiana Bertoni* extracts [7]. SCX/RPLC shows excellent performance in the 2D preparation of protein digestion [8]. In most of the 2D-HPLC preparation methods, water is usually taken as one of the mobile phase because RPLC is so widely used. For the very weak polar samples that can be hardly dissolved in aqueous solution, thus mobile phases containing water seems to be improper. A high-efficiency 2D-HPLC separation method for complex weak polar extracts from natural product by HPLC preparative columns used in nonaqueous mobile phase in both dimensional separations was quite needed. Furthermore, in the natural products area, most research objects are plants, microorganisms, and marine organisms [9–13]. Very little data of comprehensive chemical structure, pharmacology, or chromatographic methods about land higher animals bone extracts has been reported.

In this study, a bare silica column coupled with a diol column is employed to construct a novel nonaqueous 2D-HPLC separation method for the single compounds preparation from complex weak polar sample extracted from land higher animal organ. Osteon Myospalacem Baileyi extract was taken as an example to illustrate the performance of this 2D prep HPLC system.

2 Materials and methods

2.1 Apparatus and reagents

An industrial prep HPLC system was used in our laboratory. The system consists of two prep HPLC pumps, a UV detector, a sample loading pump, and a HPLC workstation (Hanbang, China). Our semipreparation was carried on Agela CHEETAH MP 100 system, which consists of two binary gradient pumps, a UV detector, a sample collector, and Agela LC software (Agela, China). Chromatographic analysis was performed on Hitachi HPLC system, which contained a Hitachi L2130 pump, Hitachi L2400 UV detector, Hitachi L2200 autosampler, and Hitachi Lachrom Elite HPLC software (Hitachi, Japan). MS and NMR spectroscopy were employed for the analysis on chemical structures. MS was performed on a Varian Saturn 2200 (Varian, USA). The NMR spectra were measured by a Bruker 600 NMR spectrometer with CD_3OD as solvent. Industrial and prep-HPLC grade ethanol and *n*-hexane were purchased from Concord Technology, China.

2.2 Sample preparation

Osteon Myospalacem Baileyi was collected from Qinghai Province and authenticated by Professor Lijuan Mei in Northwest Institute of Plateau Biology under Chinese Academy of Science. Nine kilograms of the medicinal material was powdered by an ultrafine grinder machine at −40°C to protect the chemicals in the materials from degradation. Then the superfine powder was extracted by 100 L ethanol at 60°C for 120 min three times. After filtration, the decoctions were combined and concentrated by a rotary evaporation at 60°C in vacuum. The extract was dissolved in solution of *n*-hexane/ethanol 97:3 v/v and kept for 24 h at 4°C. After centrifugation, the supernatant was filtered through 0.45 μm membranes. The final concentration of the filtered sample solution was 93.6 mg/mL.

2.3 Chromatograph condition

The first-dimension preparation was performed on a Silica prep column (150 × 250 mm id, 10 μm, 120 Å, DAISO). The mobile phase A was *n*-hexane and mobile phase B was ethanol. The linear gradient elution steps were as follows: 0–30 min, 3% B; 30–70 min, 3–10% B. The flow rate was 600 mL/min. The concentration of the loading sample was 93.6 mg/mL. The injection volume was 100 mL. Chromatograms were recorded at 214 nm. The preparation for the first-dimension purification simplified the extraction of the raw materials into fractions.

Each fraction was analyzed on DAISO Silica column (4.6 × 250 mm id, 10 μm, 100 Å, DAISO) and YMC Diol column (4.6 × 250 mm id, 10 μm, 100 Å, YMC). The mobile phase A was *n*-hexane and mobile phase B was ethanol. The flow rate was 1 mL/min. The injection volume was 10 μL. Isocratic elution procedure for Fr. 1 was 1% B for 30 min, Fr. 2 was 1.5% B for 30 min, Fr. 3 was 1.5% B for 30 min, Fr. 4 was 1.2% B for 30 min, fFr. 5 was 2% B for 30 min, Fr. 6 was 2% B for 30 min, Fr. 7 was 2.5% B for 30 min, Fr. 8 was 2% B for 30 min, Fr. 9 was 2% B for 30 min, and Fr. 10 was 3% B for 30 min. The isocratic elution procedures on both of the columns were the same. Chromatograph data were collected at 214 nm.

The second-dimension purification was performed on YMC Diol (10 × 250 mm id, 10 μm, 100 Å) column. The mobile phase A was *n*-hexane and mobile phase B was ethanol. Fractions collected from the first-dimension separation were further separated on diol column to obtain single compounds. Different isocratic elution conditions were adopted to separate fractions collected from the first dimensional preparation. Isocratic elution procedure for Fr. 1 was 1% for 30 min, Fr. 2 was 1.5% B for 30 min, for Fr. 3 was 1.5% B for 30 min, Fr. 4 was 1.2% B for 30 min, Fr. 5 was 2% B for 30 min, Fr. 6 was 2% B for 30 min, Fr. 7 was 2.5% B for 30 min, Fr. 8 was 2% B for 30 min, Fr. 9 was 2.5% B for 30 min, and Fr. 10 was 3% B for 30 min. The concentration of the loading sample was 400.0 mg/mL. The injection volume was 700 μL. The flow rate was 4 mL/min. Chromatographic data were collected at 214 nm.

Purity test of each single compound was performed on YMC Diol column (4.6 × 250 mm id, 5 μm, 100 Å, YMC). The

mobile phase A was *n*-hexane and B was ethanol. The flow rate was 1 mL/min. The injection volume was 10 μL. The linear gradient elution procedure for Fr. 1-1 and Fr. 2-1 was 1–3% B for 30 min, for Fr. 4-1, 6-1, 6-2, 6-3, and 6-4 was 1–5% B for 30 min and for Fr. 9-1 and Fr. 10–1 was 1–10% B for 30 min, respectively. Chromatographic data were collected at 214 nm.

3 Results and discussion

3.1 Establishment of 2D chromatography separation system

In the previous research on 2D-HPLC separation methods for botanical samples, the polar and weak polar fractions were all removed by SPE before the samples were loaded onto the chromatographic column [14]. SPE columns could protect the chromatographic column from dye adsorption. However, in the HPLC preparation separation research directed by pharmacology activity, no fraction was permitted to be removed before it was confirmed to be of no activity. By coincidence, in the current Osteon Myospalacem Baileyi separation work, fractions with activity were weakly polar. If we removed these weakly polar parts by SPE, the whole activity-directing separation would eventually fail. Therefore, the use of the SPE columns in traditional natural medicine separation research should be limited and more therapeutic chemicals would be found.

For the sake of constructing a 2D-HPLC preparation separation method, several columns were tested. In consideration of the weak polarity of the components in our samples, nonaqueous column seemed to be the only option for the preparative separation. Eventually, bare silica and diol columns used in nonaqueous mobile phase were selected for this work. Bare silica column was the most traditional nonaqueous normal phase column for the separation of weakly polar chemicals [15]. However, a diol column was previously usually used in aqueous HILIC mode [16]. In nonaqueous chromatography, diol column was only used for analysis [17]. No report on diol preparative column coupled with bare silica column used in nonaqueous 2D preparative chromatography could be found. Since these two columns have different stationary phase, it was possible for silica and diol column to form an orthogonal separation system.

Bare silica column was a typical example of nonaqueous normal phase. For conventional nonaqueous normal phase (NP) systems, where retention was based on surface adsorption, the following equation was found to adequately describe the retention model [18]:

$$\log k = \log k' - m'\log\varphi \quad (1)$$

Among which φ was the volume fraction of the polar solvent in the binary organic mobile phase, k' was the retention factor in pure polar solvent, and m' was the stoichiometric coefficient characterizing the number of the molecules of the strong solvent that were needed to displace one adsorbed molecule of the analyte.

In this normal phase systems model, there were three variables that affect the retention factor k, which are k', m', and φ. When the analytes and mobile phase system (*n*-hexane/ethanol) were determined, the retention factor k' in pure polar solvent could be tested and the variable k' became a constant. On the basis of the condition that analytes and mobile phase system were both determined, if the concentration of the sample solution and loading volume were invariable, the number of the molecules of the strong solvent that were needed to displace one adsorbed molecule of the analyte could also be determined, which means that the variable m' became a constant. In the condition we defined, Eq. (1) could be simplified as:

$$\log k = K_1 - M_1\log\varphi \quad (2)$$

Among which K_1 and M_1 were two constant parameters and φ was the volume fraction of the ethanol.

For HILIC systems, the retention factor k included the solvophobic and polar contributions, which depended on the polarity of the solute, chemistry of the stationary phase, and composition of the mobile phase [19]. The following equation was found to be able to adequately describe the retention model:

$$\log k = a + m_1\varphi_1 - m_2\log\varphi_1 \quad (3)$$

The parameters a, m_1, and m_2 in Eq. (3) could be determined by nonlinear regression of the experimental retention factors measured for varying volume fractions of water in the mobile phase. The variable φ_1 was the volume fraction of the polar solvent. The parameter m_1 characterized the effect of increasing the water concentration in the mobile phase on the contribution of the RP mechanism to the retention, the parameter m_2 was a measure of the opposite effect, characteristic of the HILIC contribution to the retention in highly organic mobile phases, and a is an empirical constant [20].

In the above HILIC mode, the contribution of NP and RP effects simultaneously to the retention was a rather common phenomenon, which was explained well in Eq. (3). The term $m_1\varphi_1$ was related to the interaction between solutes and solvents (RP effect). While $-m_2\log\varphi_1$ was related to the direct analyte–stationary phase interaction (NP effect). When a double organic solvent system was employed on a diol column, the interaction energy between solutes and solvents became so weak that the term $m_1\varphi_1$ became very small. Then, the term $m_1\varphi_1$ could be approximately considered as a small and non-ignorable constant. After the RP effect was minimized, NP effect became the main factor in double organic solvent system diol column chromatography. Then, the variable φ_1 became φ that was the volume fraction of the ethanol as Eq. (1) and Eq. (2). So the NP term $-m_2\log\varphi_1$ became $-M_2\log\varphi$. Eq. (3)

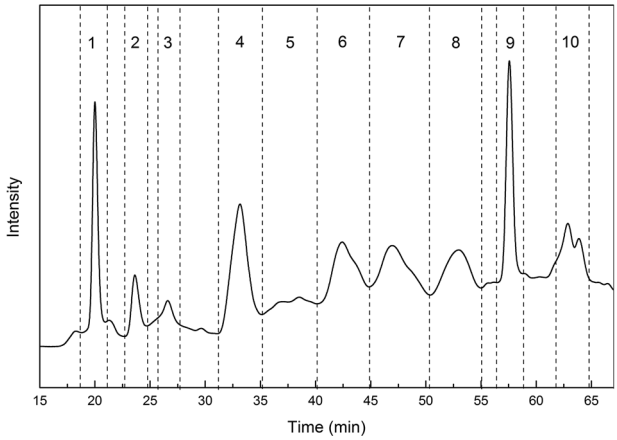

Figure 1. First-dimension preparation of crude sample on silica column (150 × 250 mm, id, 10 μm, 100 Å). The elution program was 0–30 min for 3% B, 30–70 min for 3–10% B. The flow rate was 600 mL/min. The concentration of the loading sample was 93.6 mg/mL. The injection volume was 100 mL. Chromatogram was recorded at 214 nm.

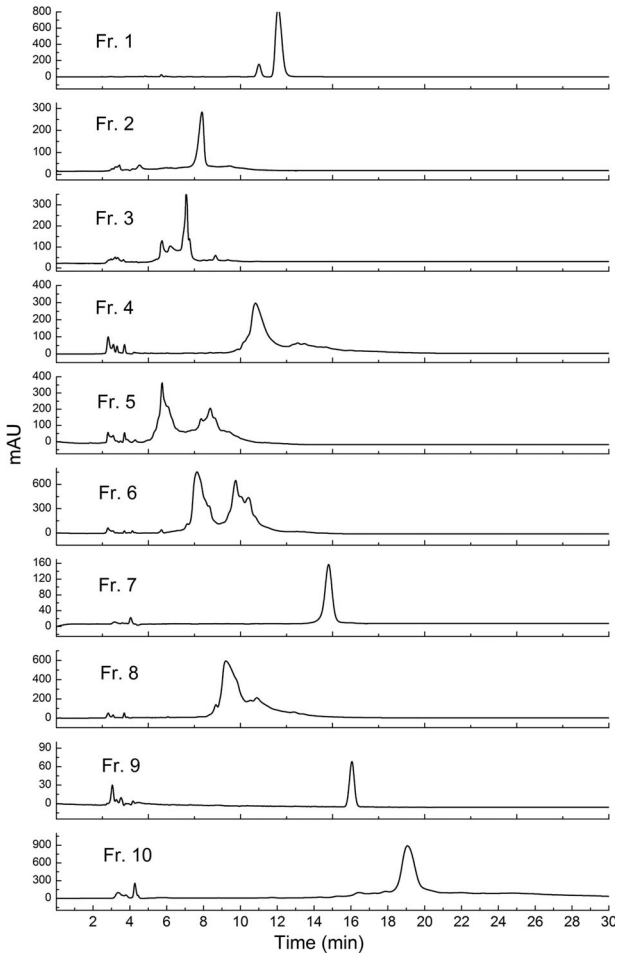

Figure 2. HPLC analysis of Fr. 1–10 on DAISO Silica column (4.6 × 250 mm, id, 10 μm, 100 Å). Isocratic elution procedure for Fr. 1 was 1% B for 30 min, Fr. 2 was 1.5% B for 30 min, Fr. 3 was 1.5% B for 30 min, Fr. 4 was 1.2% B for 30 min, Fr. 5 was 2% B for 30 min, for Fr. 6 was 2% B for 30 min, Fr. 7 was 2.5% B for 30 min, Fr. 8 was 2% B for 30 min, Fr. 9 was 2% B for 30 min, and Fr. 10 was 3% B for 30 min. Chromatogram was collected at 214 nm.

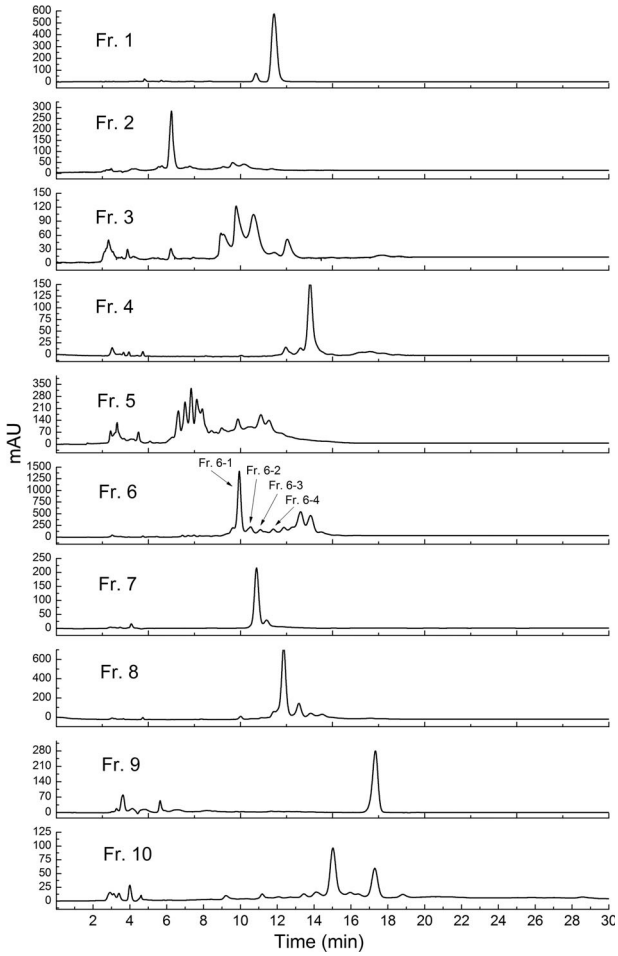

Figure 3. HPLC analysis of Fr. 1–10 on YMC Diol column (4.6 × 250 mm, id, 10 μm, 100 Å). Isocratic elution procedure for Fr. 1 was 1% for 30 min, Fr. 2 was 1.5% B for 30 min, Fr. 3 was 1.5% B for 30 min, Fr. 4 was 1.2% B for 30 min, Fr. 5 was 2% B for 30 min, Fr. 6 was 2% B for 30 min, Fr. 7 was 2.5% B for 30 min, Fr. 8 was 2% B for 30 min, Fr. 9 was 2.5% B for 30 min, and Fr. 10 was 3% B for 30 min. Chromatogram was collected at 214 nm.

could be finally simplified as the following equation in the above condition:

$$\log k = K_2 - M_2 \log\varphi \quad (4)$$

Among which K_2 was a constant that was the sum of the parameters a and the minimized term $m_1\varphi_1$, and M_2 was a constant that was a new coefficient related to analyte–stationary phase interaction. The variable φ here was the same one as φ in Eq. (2) that was the volume fraction of the ethanol in *n*-hexane/ethanol solvent system.

If we consider log$k$ as a function value and logφ as a function variable, Eqs. (3) and (4) could be turned to two linear equations that shared the same function type. The two different parameters M_1 and M_2 indicated that the sensitivities of the bare silica columns and nonaqueous diol column against mobile phase changing might be different. In addition, the retention characters of the two columns might be different too, due to their different intercepts K_1 and K_2. On the basis of

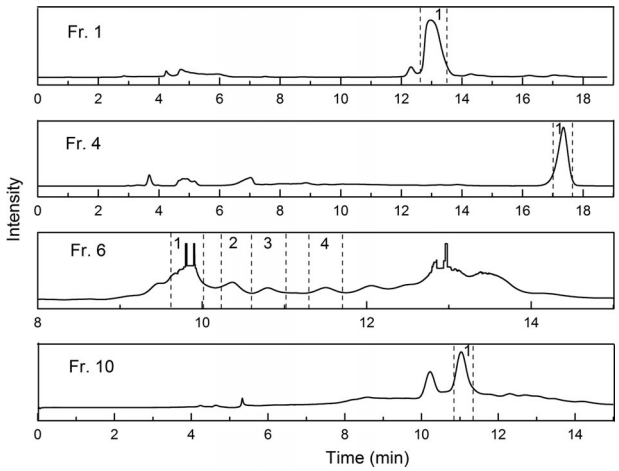

Figure 4. Second-dimension preparation of Fr. 1, 4, 6, and 10 on YMC Diol column (10 × 250 mm, id, 10 μm, 100 Å). Isocratic elution procedure for Fr. 1 was 1% for 30 min, Fr. 4 was 1.2% B for 30 min, Fr. 6 was 2% B for 30 min, and Fr. 10 was 3% B for 30 min. The concentration of the loading sample was 400.0 mg/mL. The injection volume was 700 μL. The flow rate was 4 mL/min. Chromatogram was collected at 214 nm.

the above differences between the two columns, they would have selectivity characters that were distinct enough to form an orthogonal 2D chromatography system.

3.2 First-dimension preparation

The first-dimension preparation was performed on a Silica prep column. The sample loading was 10.0 g per injection. One whole preparation procedure took 100 min, which consisted of 15 min for column balance, 15 min for column washing, and 70 min for preparation. Because of the complex composition of the crude sample, the retention times of the components differed a lot. A simple isocratic method was not enough. Gradient elution was necessary for the first dimensional preparation. Linear gradient offered the best separation performance for the components with quite different retention time. Finally, elution methods including 0–30 min for 3% B and 30–70 min for 3–10% B were used for the preparation. Preparation of the total 112.3 g crude sample took 12 injections in 21 h. The fractions were collected according to the UV absorption intensity to reduce the complexity of each fraction as much as possible. The cross in each fraction had been minimized because of the good separation repeatability. As shown in Fig. 1, ten fractions were collected in the first dimensional separation and their amounts by weight were at the range from 1.13 g for Fr. 3 to 15.44 g for Fr. 4, and the weight of most fractions were about 6.4 g. The recovery at DAISO Silica 150 × 250 mm DAC column was about 64%.

3.3 Analysis on collected fractions on DAISO silica column and YMC diol column

Fr. 1–10 were then reanalyzed on a YMC Diol column to compare the separation selectivity with the DAISO Silica column. Figures 2 and 3 showed that the elution order in these chromatograms was basically the same from each other but the intention times of the peaks were quite different, which indicated that the two columns were seasonably orthogonal as expected. Good orthogonality was significant to achieve efficient preparation of compounds with high purity from the samples. For instance, Fr. 6-1 to 6-4 could be clearly recognized on diol column. However, on silica column their retention times were so close that it was impossible to obtain satisfactory resolution. Fr. 10 was roughly a single peak on silica column and it might be regarded as a single compound in traditional technique where HPLC preparation was commonly used in the final step to yield pure compounds. In fact on diol column there were two peaks. For the coeluted compounds samples such as Fr. 10, to separate and collect very pure compounds, a good orthogonal 2D chromatography was essential rather than optional. Thus, YMC diol column would be utilized in the second dimensional preparation for its different separation selectivity, which not only improved the separation efficiency but also made a remarkable contribution to the purity of the compounds collected.

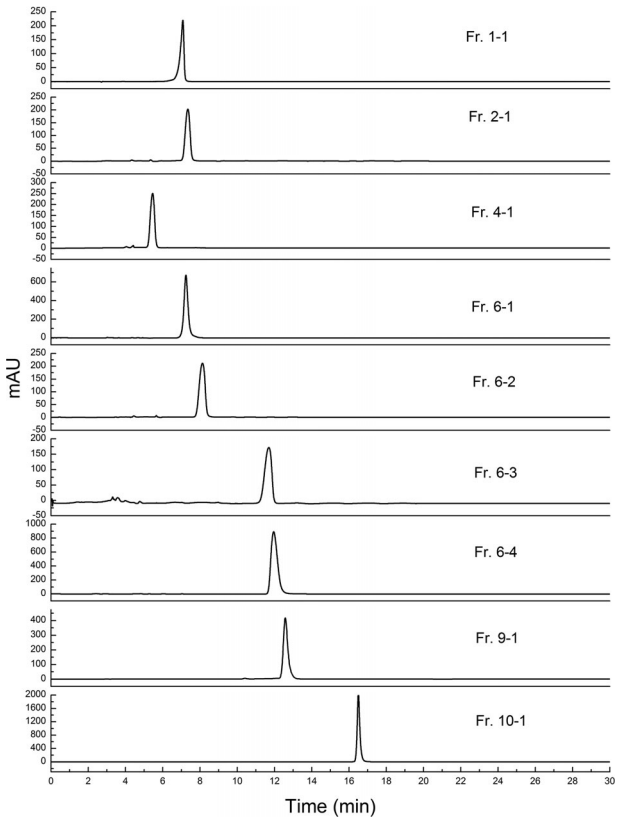

Figure 5. Purity evaluation of single compounds on YMC diol column (4.6 × 150 mm, id, 5 μm, 100 Å). The linear gradient elution procedure for Fr. 1-1 and Fr. 2-1 was 1–3% B for 30 min, for Fr. 4-1, 6-1, 6-2, 6-3, and 6-4 was 1–5% B for 30 min and for Fr. 9-1 and 10-1 was 1 to 10% B for 30 min, respectively. Chromatogram was collected at 214 nm.

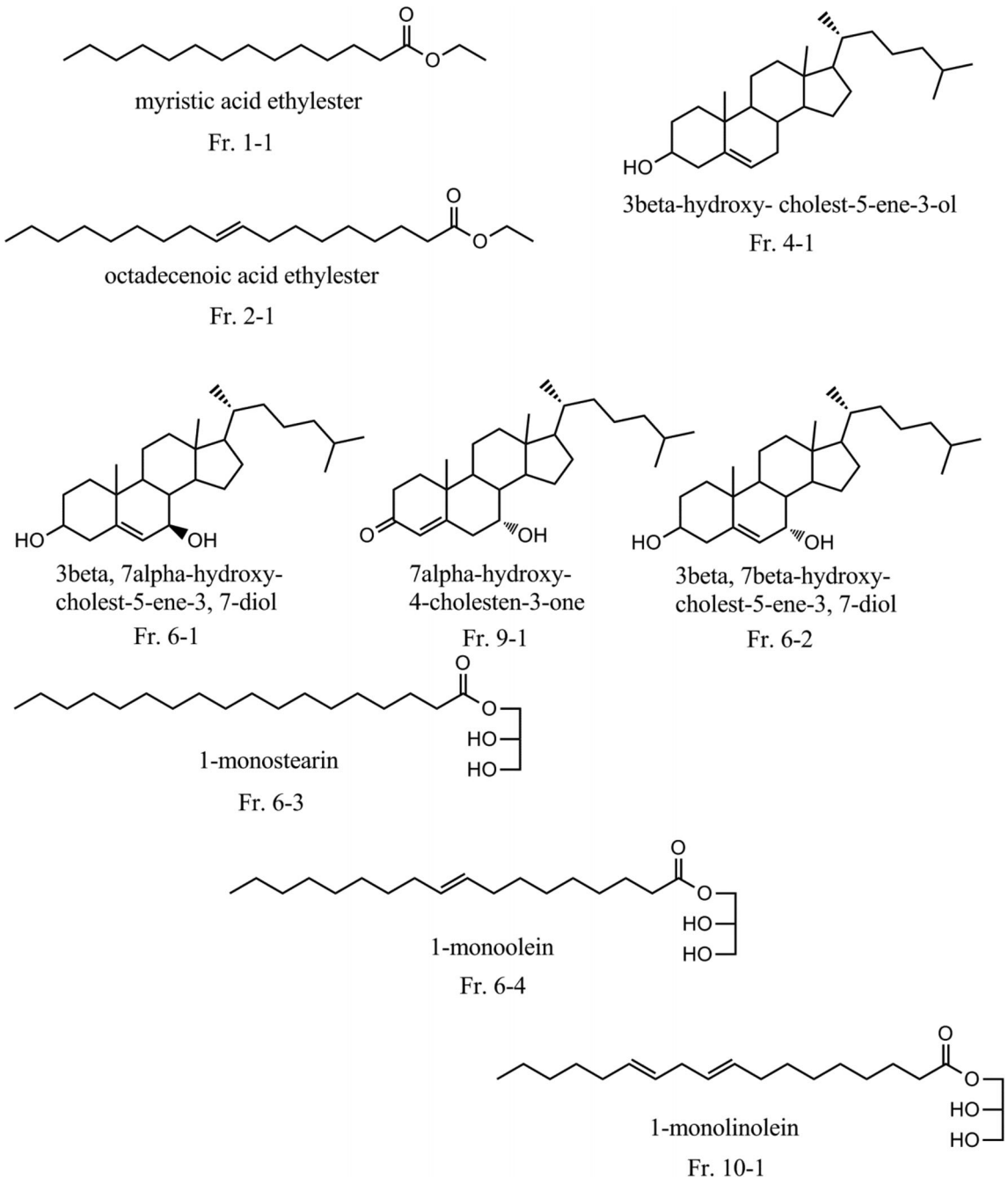

Figure 6. Structures of the purified compounds.

3.4 Preparations of the single compounds

In order to purify the compounds effectively, the separation condition was optimized for each fraction. In this method, the optimization was performed in an analytical scale. Then, the analytical scale conditions were transformed to preparative scale conditions. It was found that isocratic conditions were enough for the separation of all the fractions and isocratic elution method would avoid extra time required for reconditioning in gradient elution. The content of ethanol was optimized to make the retention time of the peaks between 5 and 25 min. After a minor revision of the elution method of the analytical conditions, the separation conditions were easily transformed to preparative scale as shown in Fig. 4. The analytical chromatograms in Fig. 3 showed similar patterns to the preparative chromatograms in Fig. 4, which demonstrated the feasibility of the transformation from an analytical scale to a preparative one. In addition, it was noteworthy that heart-cutting was used as the repeated separation strategy to insure the purity of compounds. The preparation chromatograms of

Fr. 1, 4, 6, and 10 are shown in Fig. 4. Fr. 3 and 5 were too complicated to be separated into single compounds. These two fractions were not studied in this work and are reserved for other use in the future.

After isolating Fr. 1, 4, 6, and 10, seven pure compounds were obtained and dried through rotary evaporation at 60°C in vacuum. Although the purities of Fr. 2, 7, 8, and 9 were >80% after the first-dimension preparation, it was purified in the second-dimension preparation to improve the purity. All in all, compounds **1** (Fr. 1-1, 45.2 mg), **2** (Fr. 2-1, 23.6 mg), **3** (Fr. 4-1, 207.2 mg), **4** (Fr. 6-1, 144.9 mg), **5** (Fr. 6-2, 34.4 mg), **6** (Fr. 6-3, 32.5 mg), **7** (Fr. 6-4, 15.7 mg), **8** (Fr. 9-1, 172.5 mg), and **9** (Fr. 10–1, 105.7 mg) had enough amounts to be characterized by NMR spectroscopy. In consideration of the impurities with quite different retention times, homogeneity test with isocratic elution methods was not appropriate. The ethanol content in the mobile phase was not >5% in the second dimensional preparation. Therefore, linear gradient elution methods described in Section 2.3 were enough for all the possible impurities to be tested. The purity of these compounds checked by HPLC was shown in Fig. 5 and the purity of all compounds was >98%. The structures of these compounds were shown in Fig. 6. The NMR and MS data are supplied in the Supporting Information.

4 Concluding remarks

A practical 2D preparation method was developed for the purification of high-purity compounds from higher animal bone extract. A DAISO Silica prep column was used to make fractions in the first dimensional preparation. Then, a YMC diol column used in nonaqueous mobile phases was employed to prepare compounds of high purity in the second dimensional preparation. Ten fractions obtained in the first dimensional preparation were analyzed on Silica and Diol column. The analysis result showed the good orthogonality of the 2D preparation. Benefiting from good orthogonality and optimized collection operation, nine compounds (Fr. 1-1, 45.2 mg; Fr. 2-1, 23.6 mg; Fr. 4-1, 207.2 mg; Fr. 6-1, 144.9 mg; Fr. 6-2, 34.4 mg; Fr. 6-3, 32.5 mg; Fr. 6-4, 15.7 mg; Fr. 9-1, 172.5 mg; and Fr. 10–1, 105.7 mg) with >98% purity were yielded in the second dimensional preparation. It was the first time to separate single compounds from higher animal bones by 2D preparative chromatography and this work provided some examples worthy of being referred to in future higher animal organ extract separations, such as how to dissolve sample, how to select column, and how to choose mobile phase solvent. Moreover, a novel 2D-HPLC separation system was established for the separation of weak polar complex sample. In this system, HILIC column used in nonaqueous mobile phase showed good orthogonality when coupled with traditional normal phase column. It was believed that this novel 2D-HPLC chromatography method would do much favor in the preparation of weak polar chemicals and help more useful weak polar nature chemicals to be discovered.

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The authors have declared no conflict of interest.

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