

Article

An Integrated Chromatographic Strategy for the Large-Scale Extraction of Ergosterol from *Tulasnellaceae* sp.

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Abstract: A reliable chromatographic strategy is crucial for the extraction of target compounds from natural sources as it is related to the preparation efficiency, as well as the purity of the compounds. In this study, medium-pressure normal-phase liquid chromatography and high-pressure reverse-phase liquid chromatography were combined to prepare and purify ergosterol from *Tulasnellaceae* sp. of *Gymnadenia orchidis*. First, *Tulasnellaceae* sp. was extracted three times (2.0 L and 2 h each time) with ethyl acetate, and the 6.0 L of extract solution was concentrated under reduced pressure to yield 2.2 g of crude sample. Then, the crude sample was pretreated utilizing silica gel medium-pressure liquid chromatography to enrich the target ingredient (586.0 mg). Finally, high-pressure reversed-phase liquid chromatography was used to purify the target compound, and the compound was characterized as ergosterol (purity > 95%) using spectral data. Overall, the simple and reproducible integrated chromatographic strategy developed in this study has the potential for the large-scale purification of steroids for laboratory and even industrial research. To the best of our knowledge, this is also the first report of ergosterol in *Tulasnellaceae* sp.



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Keywords: *Tulasnellaceae* sp.; *Gymnadenia orchidis*; medium-pressure liquid chromatography; reversed-phase liquid chromatography; ergosterol

1. Introduction

Natural products continue to attract scientists' attention due to their abundant sources and broad range of biological functions [1]. *Gymnosia orchidis* (also known as "Wanglag") is a kind of Tibetan medicine, which is used to treat kidney deficiency, physical weakness, and pain [2]. It is widely found in Yunnan, Tibet, Sichuan, Qinghai, Shanxi, and other places in China [3–5]. The Chinese government has currently placed it on the national second-class protected plant list. *Tulasnellaceae* sp. is one of the most significant endophytic fungi in *Gymnadenia orchidis*. Recent studies have demonstrated that ergosterol is the major component of *Gymnadenia orchidis*'s endogenous metabolite. It is a characteristic sterol of fungi, and has the functions of protecting the liver, antifibrosis, and inhibiting tumor cells. However, establishing its quality standard and evaluating its biological activity have been challenging due to a lack of adequate chromatographic purification techniques [6–8]. Therefore, the isolation of ergosterol can lay the foundation for subsequent quality control research and pharmacological activity research.

For the separation and purification of steroids (such as ergosterol) from plants, conventional methods such as thin-layer chromatography and silica gel column chromatography are commonly utilized [9–11] as these procedures only require basic equipment and are straightforward to implement. The separation of high-purity chemicals, however, continues to be problematic due to low separation repeatability and the lack of online detection

methods. High-speed countercurrent chromatography (HSCCC) has recently gained popularity for the separation of ergosterol from microbial samples and natural products [10,11], but HSCCC requires complex partition coefficient testing procedures and low separation resolution, making the separation and purification process difficult to simplify [12–14].

Preparative high-performance liquid chromatography (prep-HPLC) is an efficient strategy for the large-scale separation of individual constituents from complex samples, including microorganisms and natural products [15–18]. This method is extensively employed in numerous areas because of its excellent column efficiency, superior separation reproducibility, online detection, and automatic control [19,20]. Usually, it is worthwhile to pretreat the sample by medium-pressure liquid chromatography prior to HPLC preparation to reduce column contamination and enrich for target ingredients. Silica gel, resin, and polyamide are commonly used as sample pretreatment fillers due to their low cost and high separation selectivity [21,22]. Currently, several researchers have shown the viability of silica gel in the separation of steroids [23,24].

In view of the many reports mentioned above, in this study, we combined silica gel medium-pressure liquid chromatography with high-pressure liquid chromatography to establish a normal-phase medium-pressure/reverse-phase high-pressure mode for the efficient separation and purification of ergosterol from *Tulasnellaceae* sp. The final experimental findings revealed that such an integration strategy is economical and repeatable, and meets experimental predictions. It offers a methodological guide for subsequent attempts to produce ergosterol and its structural analogs from natural sources. Simultaneously, this is also the first description of ergosterol in *Tulasnellaceae* sp.

2. Materials and Methods

2.1. Apparatus and Reagents

An industrial-level sample pretreatment chromatographic apparatus consisting of two NP7000 pre-HPLC pumps (Jiangsu Hanbon Science & Technology, Hanbon, China), a UV detector, a manual injector (5.0 mL maximum injection volume), and an EasyChrom workstation (Jiangsu Hanbon Science & Technology) was used. HPLC analysis was carried out on a Shimadzu Essentia LC-16 instrument. The system contained a DGU-20A_{3R} degassing unit, an external column thermostat, an SPD-16 ultraviolet detector, an SIL-16 autosampler, two LC-16 pumps, and a Shimadzu workstation. MS spectra were obtained on a Waters QDa ESI mass spectrometer (Waters Corporation, Milford, MA, USA). NMR spectra were measured on a Bruker Avance 600 MHz (Bruker Corporation, Karlsruhe, Germany) with MeOH-*d*₄ as the solvent.

The column procured from Dr. Maisch & Co., Inc., Ammerbuch, Germany was a ReproSil-Pur C18 AQ (4.6 × 250 mm, 5 μm). XAmide (4.6 × 250 mm, 10 μm) and Click XION (4.6 × 250 mm, 5 μm) columns were obtained from Acchrom Technologies (Beijing, China). The Kromasil 60-5 Diol (4.6 × 250 mm, 5 μm) column used in this work was purchased from Kromasil (Bohus, Sweden). The Hypersil NH2 (4.6 × 250 mm, 5 μm) and silica (4.6 × 250 mm, 40–63 μm) columns were purchased from Dalian Elite Analytical Instrument (Liaoning, China).

Chromatographic-grade methanol, ethyl acetate, and dichloromethane used for HPLC were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China); analytical-grade dichloromethane and methanol were purchased from Yunnan Xinlanjing Chemical Industry, Qujing, China.

2.2. Sample Preparation

Roots of *Gymnadenia orchidis* were collected from Maixiu farm, Tongren county, Xining, China, in August 2017, and validated by professor Pengcheng Lin of the College of Pharmacy, Qinghai Nationalities University. A sample (No. 2017-L-3) was stored in the Qinghai Nationalities University Museum of Biology. *Tulasnellaceae* sp. was separated from the roots of *Gymnadenia orchidis*, and the identification of the *Tulasnellaceae* sp. is given in the File S1 of supporting information.

Tulasnellaceae sp. solid fermentation extracellular metabolites (32.5 g) were extracted thrice at room temperature with ethyl acetate (2.0 L and 2 h each). The combined extracted solution (6.0 L) was filtered and concentrated at 40 °C using a rotary evaporator to obtain the crude sample (2.2 g). The crude sample was dissolved in dichloromethane (6.0 mL) and then wet-loaded onto a silica gel medium-pressure column (40–63 µm, 49 mm × 460 mm, SiliCycle, Quebec, Canada). Methanol/dichloromethane were used as the eluent and the linear gradient was set to 0–30 min, 0–35% methanol, and 59.0 mL/min flow rate at room temperature. The chromatogram was recorded at 270 nm. After one separation, the collected eluate solution was concentrated and dried to finally obtain the target fraction (fraction 2, 586.0 mg).

2.3. Chromatographic Conditions

The crude sample and fraction 2 were analyzed on five representative analytical columns (Silica, XAmide, Hypersil NH₂, Kromasil 60-5 Diol, and Click XION). The crude sample (2.2 g) was dissolved in 6.0 mL of dichloromethane and passed through a 0.45 µm filter to obtain the sample solution (367.0 mg/mL). Fraction 2 (12.0 mg) was dissolved in 2.0 mL of methanol and passed through a 0.45 µm filter. The isocratic elution procedure was 100% dichloromethane for 16 min, the injection volume was 1.0 µL, and column temperature was maintained at room temperature. Chromatograms were recorded at 270 nm.

The fraction 2 solution (6.0 mg/mL) was analyzed on a ReproSil-Pur C18 AQ analytical column. The isocratic elution procedure was 95% methanol for 40 min, the injection volume was 1.0 µL, and the column temperature was maintained at room temperature.

The purity of fraction 2-2 was analyzed on a ReproSil-Pur C18 AQ analytical column, and the isocratic elution procedure was 100% methanol for 40 min with a 1 mL/min flow rate at room temperature. The monitoring wavelength was 270 nm.

3. Results and Discussion

3.1. Extraction and Analysis of Endogenous Metabolites from *Gymnadenia orchidis*

A total of 2.2 g of crude sample (6.8% yield) was obtained from 32.5 g of *Tulasnellaceae* sp. solid fermentation extracellular metabolites, which were then all dissolved in 6.0 mL of dichloromethane for subsequent analysis and pretreatment. The literature reveals that the structural characteristics and chemical properties of ergosterol give it a unique chromatographic retention behavior [25]. Therefore, the choice of chromatographic column filler is essential to obtain high-purity ergosterol from liquid chromatography.

To determine the optimal conditions, the chromatographic behavior of five representative chromatographic columns (Silica, XAmide, Hypersil NH₂, Kromasil 60-5 Diol, and Click XION) against the crude sample was investigated in normal phase mode. Isocratic elution of 100% dichloromethane was used on these columns with an injection volume of 1.0 µL at room temperature. The analysis result is shown in Figure 1. We observed that the crude sample showed up as two main fractions (fraction 1 and fraction 2) and exhibited good resolution on all five chromatographic columns (Figure 1A–E). Previous literature reports confirmed that ergosterol is the main component of the endogenous metabolite from *Gymnadenia orchidis*; therefore, fraction 2 was considered the target fraction for further purification. Medium-pressure liquid chromatography pretreatment of the crude extracts of natural products can remove some nontarget ingredients (natural pigments, macromolecular polymers, etc.), enrich target components, and simplify the workload of preparative high-pressure liquid chromatography. Silica gel is often used as filler for medium-pressure liquid chromatography pretreatment because of its low cost and high separation selectivity. As shown in Figure 1, all five chromatographic columns have the potential for pretreatment because of their effective separation of the crude sample. After comprehensive consideration of the quick peak flowing out, convenience, and moderate price, silica gel medium-pressure liquid chromatography was chosen for the pretreatment of the crude sample.

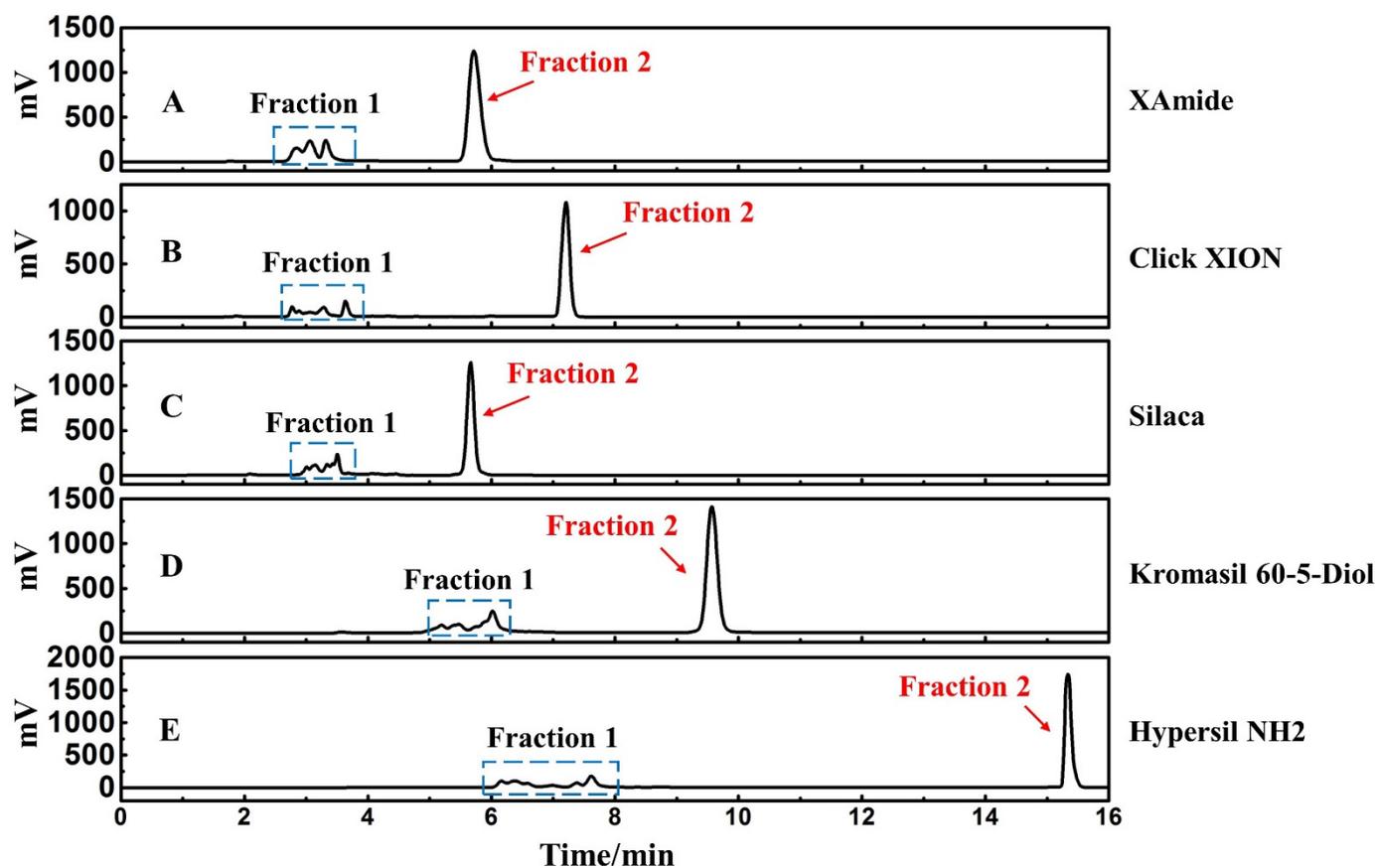


Figure 1. HPLC analysis of crude sample on five representative chromatography columns ((A): XAmide, (B): Click XION, (C): Silica, (D): Kromasil 60-5 Diol, and (E): Hypersil NH₂) under normal phase mode; injection volume: 1.0 μ L. Conditions: isocratic elution: 0–16 min, 100% dichloromethane; monitoring wavelength: 270 nm; flow rate: 1.0 mL/min; column temperature: 30 $^{\circ}$ C.

3.2. Crude Sample Pretreatment via Medium-Pressure Chromatography

On the basis of the analysis results in Section 3.1, silica gel medium-pressure chromatography was used for the pretreatment of the crude sample. The supernatant liquid (6.0 mL) was removed from the top of the medium-pressure chromatographic tower, and the crude sample solution (6.0 mL) was added to the top of the medium-pressure chromatographic tower. Then, the medium-pressure chromatographic tower was connected to the preparative liquid chromatography for the pretreatment of the crude extract. The separation chromatogram is shown in Figure 2. Fraction 1 was nontarget ingredients, and fraction 2 was the target fraction for pretreatment. Due to the increase in the loading volume and the column diffusion effect, the retention time of the target component and peak shape were slightly different compared with the analytical chromatogram; however, it did not affect the recovery of fraction 2, and the visual separation of fraction 1 and fraction 2 was still achieved. After one separation, fraction 2 (586.0 mg) was obtained after concentration under reduced pressure, with a recovery of 26.6%. Fraction 2 (12.0 mg) was dissolved in 2.0 mL of methanol solution and passed through a 0.45 μ m filter for subsequent analysis and separation.

3.3. Further Purification of Fraction 2 on ReproSil-Pur C18 AQ Chromatographic Column

Fraction 2 was analyzed on an XAmide and a ReproSil-Pur C18 AQ analytical chromatographic column. The result is shown in Figure 3. At the same time, the analysis results of the crude sample on the XAmide chromatographic column are also listed together for comparison (Figure 3B). Figure 3A,B demonstrates an excellent pretreatment effect; however, when the analysis was performed on a different selective column (Figure 3C), it was found that fraction 2 showed multiple low peaks (nontarget ingredients) and one

main peak (target ingredient). We speculated that silica gel medium-pressure normal-phase liquid chromatography was used for the pretreatment of the crude sample, which has a natural separation selectivity different from the ReproSil-Pur C18 AQ column (reversed-phase chromatographic column). In normal phase mode, some components with consistent retention times of the target peaks cannot be visually separated; however, this problem is often well-solved in inverse phase mode because of the characteristic of selective variability [26–28]. Therefore, the ReproSil-Pur C18 AQ column was ultimately selected to further purify fraction 2.

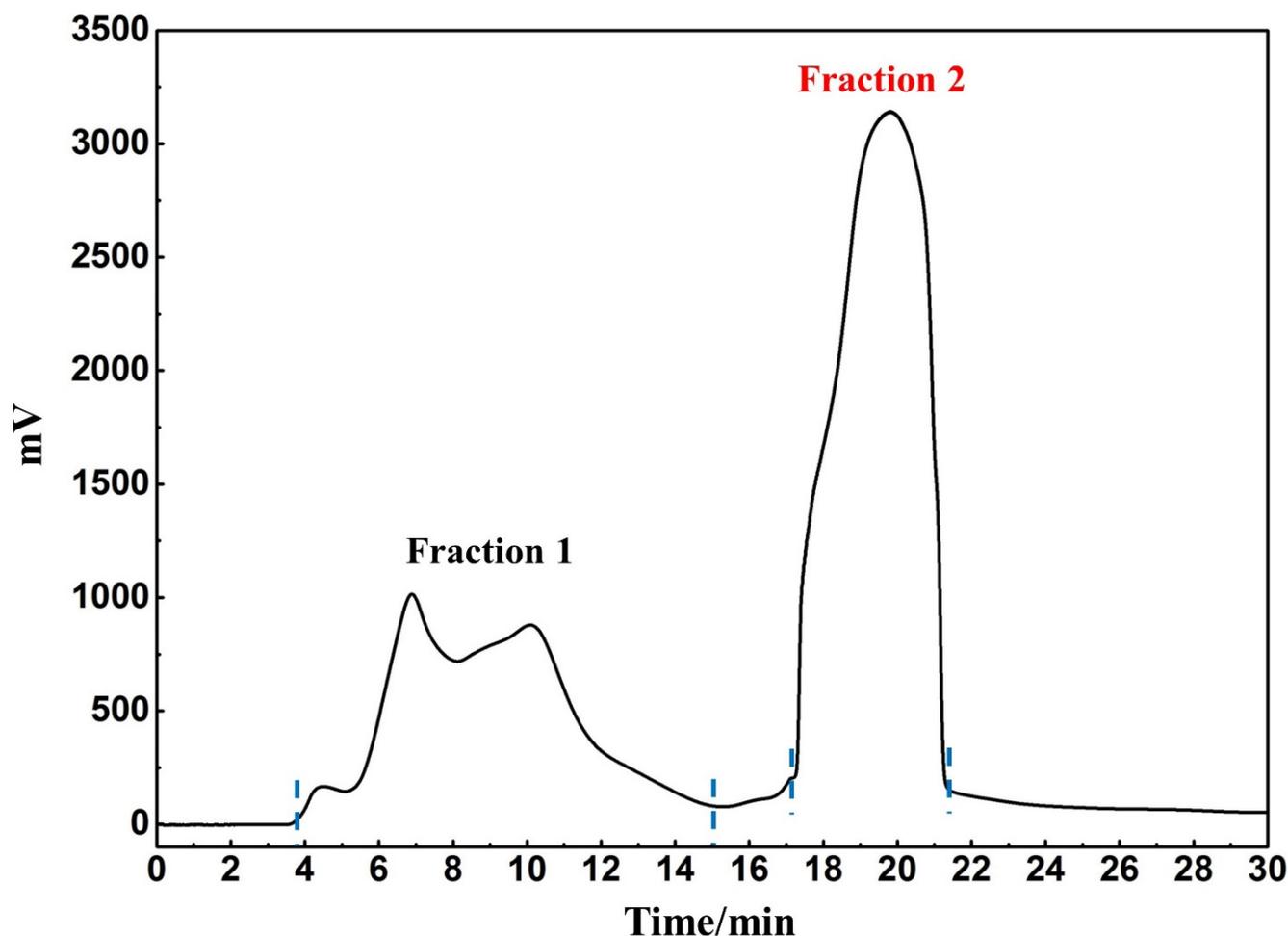


Figure 2. Pretreatment chromatogram of target components in *Tulasnellaceae* sp. with silica gel medium-pressure chromatographic tower; injection volume: 6.0 mL. Conditions: mobile phase A: dichloromethane, B: methanol; gradient: 0–30 min, 0–35% B; monitoring wavelength: 270 nm; flow rate: 59.0 mL/min.

3.4. Structural Characterization of Ergosterol

Fraction 2 (2.0 mL) solution was subjected to further isolation and purification. The loading volume for each preparation was 0.96 mL, and the eluate was concentrated under reduced pressure after 12 cycles of preparation. Figure 4A shows the comparison diagram for preparations, showing that the sample loading volume was large and reproducible and meets the needs of large-scale preparation. Finally, a total of 5.9 mg of compound was obtained with a recovery rate of 49.2%. The purity of the isolated compound was examined using a ReproSil-Pur C18 AQ analytical column. The chromatographic conditions are described in Section 2.3. As shown in Figure 4B, fraction 2-1 was obtained with >95% purity.

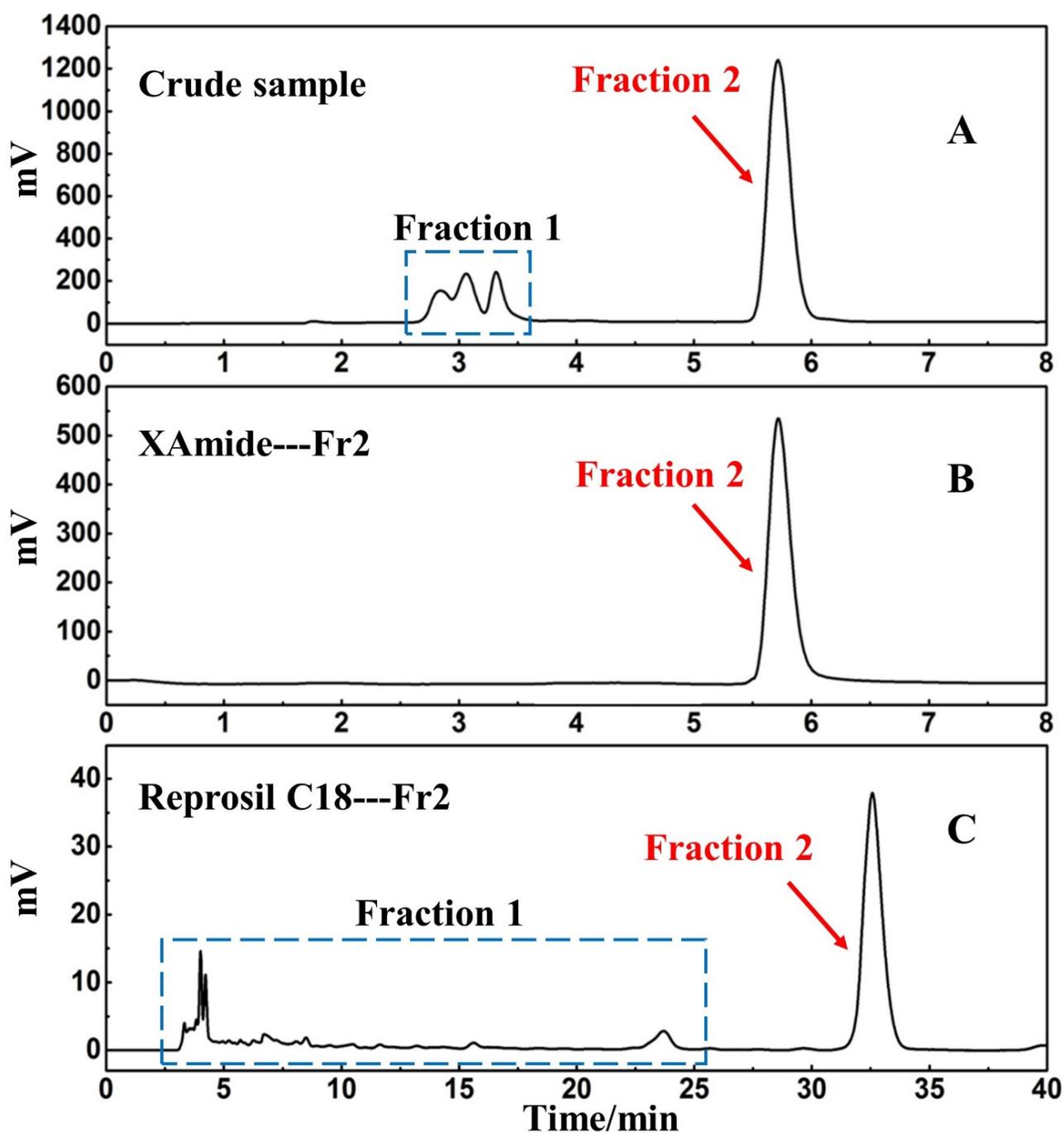


Figure 3. HPLC analysis of the target fraction on XAmide ((A): crude sample; (B): Fraction 2) and Reprosil C18 ((C): Fraction 2) analytical column; injection volume: 1.0 μ L. Conditions of XAmide analytical column: isocratic elution: 0–16 min, 100% dichloromethane; monitoring wavelength: 270 nm; flow rate: 1.0 mL/min; column temperature: 30 $^{\circ}$ C. Conditions of Reprosil C18 analytical column: isocratic elution: 0–40 min, 100% methanol; monitoring wavelength: 270 nm; flow rate: 1.0 mL/min; column temperature: 30 $^{\circ}$ C.

To elucidate the structure of fraction 2-1, ESI-MS, ^1H NMR and ^{13}C NMR spectra were obtained and compared with published literature data. From the spectral data, we concluded that fraction 2-1 corresponded to ergosterol [6,29]. Original spectra are shown in the Figures S1–S3 of supporting information. The chemical structure of ergosterol is shown in Figure 4B.

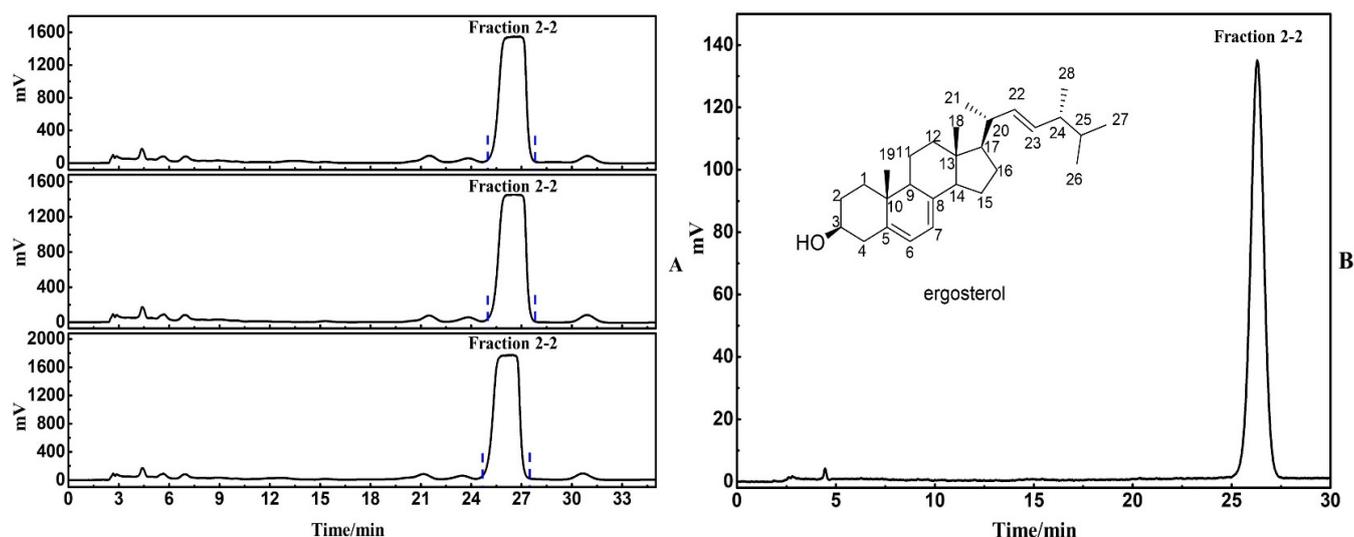


Figure 4. The preparative chromatogram and HPLC purity assay of the target fraction on ReproSil C18 analytical column in methanol/water (A). Conditions: mobile phase water and methanol; isocratic elution: 0–35 min, 100% methanol; monitoring wavelength: 270 nm; flow rate: 1.0 mL/min; injection volume of the preparative chromatogram: 80.0 μ L; injection volume of HPLC purity assay: 1.0 μ L; column temperature: 30 $^{\circ}$ C. The purity of the isolated compound was examined using a ReproSil-Pur C18 AQ analytical column (B). The isocratic elution procedure was 100% methanol for 40 min with a 1 mL/min flow rate at room temperature. The monitoring wavelength was 270 nm.

Fraction 2-2 (ergosterol, 5.9 mg, white powder, ESI-MS m/z : 398.34 $[M+2H]^+$): 1H NMR (600 MHz, $CDCl_3$) 5.57 (1H, dd, $J = 5.6, 2.5$, H-6), 5.38 (1H, t, $J = 5.4$, H-7), 5.20 (1H, dd, $J = 15.3, 7.5$ Hz, H-22), 5.20 (1H, dd, $J = 15.3, 7.5$ Hz, H-23), 3.64 (1H, s, H-3), 1.179–2.502 (H-1, H-2, H-4, H-9, H-11, H-12, H-14, H-15, H-16, H-17, H-20, H-24, H-25), 1.04 (3H, d, $J = 6.6$, 21-Me), 0.95 (3H, s, 19-Me), 0.92 (3H, d, $J = 6.9$, 28-Me), 0.83 (6H, d, $J = 6.9$ Hz, 26-Me, 27-Me), 0.83 (6H, d, $J = 6.9$ Hz, 26-Me, 27-Me), 0.63 (3H, s, 18-Me); ^{13}C NMR (151 MHz, $CDCl_3$), 141.4 (C-8), 139.8 (C-5), 135.6 (C-22), 132.0 (C-23), 119.6 (C-6), 116.3 (C-7), 70.5 (C-3), 55.7 (C-17), 54.6 (C-14), 46.2 (C-9), 42.8 (C-13), 42.8 (C-24), 40.8 (C-4), 40.4 (C-20), 39.1 (C-12), 38.4 (C-1), 37.0 (C-10), 33.1 (C-25), 32.0 (C-2), 28.3 (C-16), 23.0 (C-15), 21.1 (C-11), 21.1 (C-21), 19.9 (C-26), 19.6 (C-27), 17.6 (C-28), 16.3 (C-19), 12.0 (C-18).

4. Conclusions

In this study, silica gel medium-pressure normal-phase liquid chromatography coupled with high-pressure reverse-phase liquid chromatography was developed for the large-scale purification of ergosterol from extracellular metabolites of *Tulasnellaceae* sp. First, the metabolite (2.2 g) of *Tulasnellaceae* sp. was pretreated by silica gel medium-pressure normal-phase chromatography to obtain fraction 2, and then the target compound was directly obtained after purification of fraction 2 using a ReproSil-Pur C18 AQ column, which was identified as ergosterol. Experiments showed that medium-pressure silica gel is well-suited for the pretreatment of natural products. Meanwhile, the medium-pressure normal-phase/high-pressure reverse-phase mode used in this study was able to separate ergosterol efficiently and on a large scale, which is expected to be further developed and applied in future methodological studies for the separation from natural products.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations9070176/s1>, File S1: Species identification of the endophytic fungus *Tulasnellaceae* sp. From *Gymnadenia orchidis*, Figure S1: ESI mass spectrum of ergosterol, Figure S2: 1H NMR spectrum (600 MHz) of ergosterol on ReproSil C18 analytical column in water/ethanol (in $CDCl_3$); Figure S3: ^{13}C NMR Spectrum (151 MHz) of ergosterol on ReproSil C18 analytical column in water/ethanol (in $CDCl_3$).

Author Contributions: Conceptualization, J.D. and P.L.; methodology, J.D. and Z.W.; formal analysis, Z.W. and Q.W.; resources, J.Z.; writing—original draft preparation, Z.W.; writing—review and editing, Z.W., J.D. and P.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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