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Combination of Medium- and High-Pressure Liquid Chromatography for Isolation of L-tryptophan (Q-marker) from *Medicago sativa* Extract

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Abstract: *Medicago sativa* (alfalfa) is a widely used animal feed. However, its quality has been difficult to control due to the lack of appropriate marker compounds. Therefore, it is very necessary to select an appropriate quality marker (Q-marker) to control its quality. In this study, medium-pressure liquid chromatography and high-pressure liquid chromatography were employed to effectively prepare the separation of the Q-marker (L-tryptophan) from *Medicago sativa*. Firstly, using MCI GEL® CHP20P as the stationary phase, 2.5 g of the target fraction Fr3 was enriched from crude *Medicago sativa* extract (2.9 kg) by medium-pressure liquid chromatography. Secondly, Sephadex LH-20 was used to further separate Fr3 fractions, and the Fr34 fraction (358.3 mg) was enriched after 14 repetitions. Lastly, using the ReproSil-Pur C18 AQ preparative column, 63.4 mg of L-tryptophan was obtained by high-pressure liquid chromatography, and the purity was above 95%. The results showed that medium-pressure liquid chromatography (MCI GEL® CHP20P and Sephadex LH-20) combined with high-pressure liquid chromatography (ReproSil-Pur C18 AQ) could be used to effectively prepare the Q-marker from natural products with satisfactory purity.

Keywords: *Medicago sativa*; medium-pressure liquid chromatography; high-pressure liquid chromatography; quality marker; L-tryptophan

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1. Introduction

Medicago sativa (alfalfa), called "Queen of the forages", is a widely cultivated perennial leguminous plant used as animal feed [1,2]. It is mainly cultivated in temperate regions of the northern and southern hemispheres, with an area of about 32 million hectares [3]. Firdaous et al. studied compounds of *Medicago sativa* and found that they were beneficial to the feed industry [4]. Because of its high protein content and the full range of amino acids, it was a good source of available protein and amino acids for livestock [5–7]. In addition, *Medicago sativa* cultivation required low inputs of herbicides and pesticides. This was the reason for the choice of *Medicago sativa* as the main commercially grown source of forage and fodder in many countries [8]. These countries not only focused on yield, but the quality of *Medicago sativa* was also an important factor for them to consider. However, there were many factors that can affect the quality of *Medicago sativa*, including soil moisture, ambient temperature, and mode of transportation, making the quality difficult to control [9–11]. Therefore, it was necessary to choose an appropriate quality standard to control its quality. However, there was no related research on the quality control system of *Medicago sativa*. The quality control index of traditional Chinese medicine is Q-marker. As

the main component, Q-marker could reflect the safety and efficacy of traditional Chinese medicine [12,13]. Because the quality control system of Chinese medicine and forage is similar, this study hence focused on isolating the key indicator of quality control of *Medicago sativa*—Q-marker.

The conventional method for preparation, separation, and purification was silica-gel column chromatography because of its cheapness and simple operation [14–16], whereas this method still had the problem of poor reproducibility, instability, and low recovery [17,18]. Another technique was high-speed countercurrent chromatography (HSCCC), which was utilized for preparative separation and purification of diverse natural products [19]. This liquid–liquid partition chromatography technology, unaffected by solid support matrices, could eliminate the irreversible adsorption of samples onto stationary phases that occurs with traditional column chromatography [20,21]. Nonetheless, there were significant drawbacks to this approach, such as low separation resolution, the ability to separate only moderately polar molecules, and the requirement to calculate partition coefficients.

Many laboratories have used high-pressure liquid chromatography to isolate milligram-scale target compounds. Dang et al. used this method to prepare and isolate gallic acid derivatives and 1,1-diphenyl-2-picrylhydrazine inhibitors from *Saxifraga tangutica*. Vichapong et al. used HPLC to optimize the conditions for the extraction of phenolic compounds from rice [22–25]. Hence, the Q-marker could be separated and quantitatively determined by high-pressure liquid chromatography. However, high-pressure liquid chromatography carried the risk of contamination of the stationary phase, for example, chlorophyll in plants adsorbs to the stationary phase [26]. Therefore, a technique for pretreatment of the crude sample was required to remove non-target compounds. Due to medium-pressure liquid chromatography having the advantages of automation, high reproducibility of separation, and durable stationary phase materials, it was widely used for the enrichment of high-purity compounds [27–30]. Therefore, medium-pressure liquid chromatography could be used to remove non-target compounds. The stationary phase materials included various forms of silica gel, MCI GEL® CHP20P, and Sephadex LH-20 [22,31,32]. On this basis, the combination of medium-pressure liquid chromatography with different stationary phases could better enrich the target compounds [33]. As a result, for sample preparative separation, we used two steps of medium-pressure liquid chromatography in conjunction with high-pressure liquid chromatography. This method could not only solve the problem of easy contamination of the high-pressure liquid chromatography stationary phase but also separate higher-purity compounds through different stationary phases.

The aim of this research was to utilize a combination of high-pressure liquid chromatography and medium-pressure liquid chromatography to extract L-tryptophan in *Medicago sativa* and use it as a Q-marker for this forage grass. First, MCI GEL® CHP20P and Sephadex LH-20 (Mitsubishi Chemical Corporation, Tokyo, Japan) were used as stationary phases for medium-pressure liquid chromatography to enrich the target fractions. Subsequently, a ReproSil-Pur C18 AQ (Hanau, Hessen, Germany) high-pressure preparative column was used to purify the target fractions. This method could be used to determine the Q-marker of other crops and establish the quality control system.

2. Materials and Methods

2.1. Equipment and Chemicals

The preparative medium-pressure liquid chromatography workstation (Hanbon Science & Technology Co., Ltd., Jiangsu, China) consisted of two NP7000 prep-high-performance liquid chromatography pumps, an NU3000 UV–Vis detector, a 5 mL manual injector, and an LC workstation. High-performance liquid chromatography with an Essentia LC-16 (Shimadzu Instruments Co., Kyoto, Japan) was used to analyze the samples. This system consisted of two binary gradient pumps, a UV–Vis detector, a 5 mL manual injector, and an LC workstation. ESI-MS spectra were acquired as mass spectrometer instruments. 1H and 13CNMR spectra

were recorded on the Bruker Avance 600 MHz spectrometer (Bruker Instruments, Karlsruhe, Germany) with DMSO-*d*6 as the solvent.

The separation material MCI GEL®CHP20P (120 μm) and Sephadex LH-20 were purchased from Mitsubishi Chemical Corporation (Tokyo, Japan) and GE Healthcare (Chicago, IL, USA), respectively. The analytical column ReproSil-Pur C18 AQ (4.6 × 250 mm, 5 μm) and ReproSil-Pur C18 AQ preparative column (20 × 250 mm, 5 μm) were purchased from Maisch Corporation (Hanau, Hessen, Germany).

The chromatographic and analytical-grade methanol (MeOH) was bought from Kelon Chemical Reagent Factory (Chengdu, China). Chromatographic-grade water was prepared by Moore water purification station (Chongqing, China).

2.2. Preparation of Extract of Medicago Sativa

The whole *Medicago sativa* was planted in the Northwest Plateau Institute of Biology and validated by Prof. Lijuan Mei of Northwest Institute of Plateau Biology. A sample had been stored in the Key Laboratory of Tibetan Medicine Research, the Chinese Academy of Sciences.

The dried *Medicago* sativa was cut into 1 cm pieces (2.9 kg) and extracted twice with 25.0 L MeOH (12 h each time). The 50.0 L extract was collected, filtered, and concentrated with a 45 °C rotary evaporator. After the extract was concentrated to 10.0 L, 1.0 mL of the extract was taken out and analyzed on a ReproSil-Pur C18 AQ analytical column (4.6 × 250 mm, 5 μm). With 0.1% v/v formic acid in water as mobile phase A and methanol as mobile phase B, the flow rate was 1.0 mL/min. The linear gradient elution settings were as follows: 0–50 min, 0–100% B; 50–60 min, 100% B. An amount of 920.0 g of amorphous silica gel was added to the remaining concentrated solution and dried in an oven at 40 °C. Finally, 1.2 kg of a mixture of dried silica gel and the sample was obtained (262.4 g of the sample).

2.3. Medium-Pressure Liquid Chromatography Sample Pretreatment

The dried silica gel mixture (104.4 g) was placed in another small medium-pressure chromatogram column (49 × 100 mm) and connected to a medium-pressure column (49 × 460 mm) equipped with MCI GEL® CHP20P as a stationary phase, and a MeOH/H2O combination was used for elution. Chromatographic pure water was mobile phase A, and methanol was mobile phase B. The linear elution gradient was: 0–40 min, 0% B; 40–340 min, 0–100% B; 340–540 min, 100% B. During this process, the flow rate was kept constant at 50.0 mL/min. The chromatogram record was obtained at 210 nm. After 11 repetitions, 12 fractions were obtained, which were rotary evaporated and weighed. The final fraction, fraction 3 (Fr3), was selected for further enrichment.

Fr3 (30.0 mg) was dissolved in methanol and analyzed on Resilpur C18 AQ (20 × 250 mm, 5 μm), and 0.1% v/v formic acid in water was used as mobile phase A, methanol solution was used as mobile phase B, and the flow rate was 1.0 mL/min. Linear gradient elution settings were as follows: 0–50 min, 0–100% B; 50–60 min, 100% B. Subsequently, the conditions were further optimized, and the optimized conditions were: 5–65% MeOH for 60 min at a flow rate of 1.0 mL/min.

The remaining Fr3 (70.0 mg per injection, 978.7 mg total weight) was dissolved in methanol and added to the medium-pressure chromatography column equipped with Sephadex LH-20. It was eluted with MeOH for 5000 min, with a flow rate of 0.3 mL/min at 210 nm. After repeating the separation 14 times, fraction 34 (Fr34) was collected, combined, and then concentrated by rotary evaporation and dried and weighed (538.3 mg). Fr34 was analyzed with ReproSil-Pur C18 AQ (20 × 250 mm, 5 μm) analytical column. The chromatographic conditions: mobile phase A was 0.1% v/v formic acid in water, mobile phase B was chromatographic methanol, the gradient elution condition was 0–60 min, 5–65% B, the flow rate was 1.0 mL/min, and detection wavelength was 210 nm.

2.4. High-Pressure Liquid Chromatography Purification Quality Marker

The ReproSil-Pur C18 AQ analytical column (4.6 × 250 mm, 5 µm) was used to re-analyze Fr34. Fr34 (538.3 mg) was dissolved and filtered through a 0.45 µm membrane. Mobile phase A was 0.1% *v*/*v* formic acid in water, and mobile phase B was methanol; the isocratic elution condition was 0–60 min 8% B, the flow rate was 1.0 mL/min, and the detection wavelength was 210 nm.

After analysis, the ReproSil-Pur C18 AQ preparative column (20 × 250 mm, 5 µm) was used to separate Fr34. The mobile phase is composed of methanol and 0.1% *v*/*v* formic acid in water. Before separation, the system was equilibrated for 10.0 min followed by isocratic elution with 8% methanol for 60 min. Each test is based on injecting 0.5 mL of Fr34 sample solution. The flow rate of the eluent is continuously maintained at 19.0 mL/min, and the elution process is tracked at 210 nm. Finally, 62.6 mg of fraction 341 (Fr341) was prepared.

2.5. Purity Evaluation of the Obtained Quality Marker

The purity of the separated Fr341 was evaluated using ReproSil-Pur C18 AQ analytical column (4.6 × 250 mm, 5 µm). The mobile phases were 0.1% *v*/*v* formic acid in water (A) and methanol (B). The isocratic elution was based on 8% B, the flow rate was 1.0 mL/min, the elution time was 60 min, and the absorbance was 210 nm.

The ReproSil-Pur C18 AQ analytical column (4.6 × 250 mm, 5 µm) was used to analyze Fr341 and the *Medicago sativa* crude sample. Mobile phase A was 0.1% *v*/*v* formic acid in water, and mobile phase B was methanol. The linear gradient elution was based on 0–60% B, the flow rate was 1.0 mL/min, the elution time was 60 min, and the absorbance was 210 nm.

3. Results

3.1. Medium-Pressure Chromatography for Enrichment of the Quality Marker Faction

An analytical ReproSil-Pur C18 AQ column (4.6 × 250 mm, 5 µm) was used to examine a crude sample of *Medicago sativa* extracted with MeOH. The extract includes the main component (approximately 17 min peak), as shown by the analytical chromatogram in Figure 1A. It is evident from the analytical chromatogram in Figure 1A that the crude MeOH extract of *Medicago sativa* contains a main compound. About 10.0 L of the concentrated extract obtained by rotary evaporation was mixed with silica gel (781.6 g, 100–200 mesh), dried, and weighed. About 262.4 g of crude sample was obtained from 2.9 kg of *Medicago sativa* in a yield of 9.2%. Before the MCI GEL® CHP20P pretreatment, the dried silica gel combination (104.4 g) was put in another small medium-pressure chromatographic tower (49 × 100 mm). The connection of the medium-pressure liquid chromatography was shown in Figure 1D, connecting two medium-pressure columns (49 × 100 mm and 49 × 460 mm) to the preparative liquid chromatography. After 11 repetitions, the crude extract was separated into 12 fractions (Fr1–Fr12) by MCI GEL® CHP20P medium-pressure liquid chromatography, yielding the chromatogram in Figure 1B. The ReproSil-Pur C18 AQ analytical column was used to analyze Fr3 (the same mobile phase and chromatographic conditions as the crude sample), resulting in the chromatogram in Figure 1C.

For better separation of target compounds from complex crude extracts, the elution conditions of Fr3 needed to be optimized before further preparation at medium-pressure liquid chromatography. Subsequently, the analysis was performed on a ReproSil-Pur C18 AQ analytical column with the optimized conditions to obtain the analytical chromatogram in Figure 2A. The red-hearted marked peak in this chromatogram was the main compound's peak. As shown in Figure 2D, the medium-pressure column (26 × 1000 mm) filled with separation material Sephadex LH-20 was connected to preparative liquid chromatography. The Fr3 (70.0 mg) sample was dissolved with 3 mL methanol and injected on a Sephadex LH-20 medium-pressure liquid chromatography column. It was eluted with isocratic MeOH for 5000 min at a flow rate of 0.3 mL/min. The resulting chromatogram is shown in Figure 2B, and six fractions (Fr31–Fr36) were obtained. Fr34 as the main fraction was collected, concentrated, and weighed (358.3 mg), with 17.0% recovery. Subsequently,

Fr34 was analyzed using the same chromatographic conditions as the optimized Fr3 on a ReproSil-Pur C18 AQ analytical column, resulting in the chromatogram in Figure 2C.

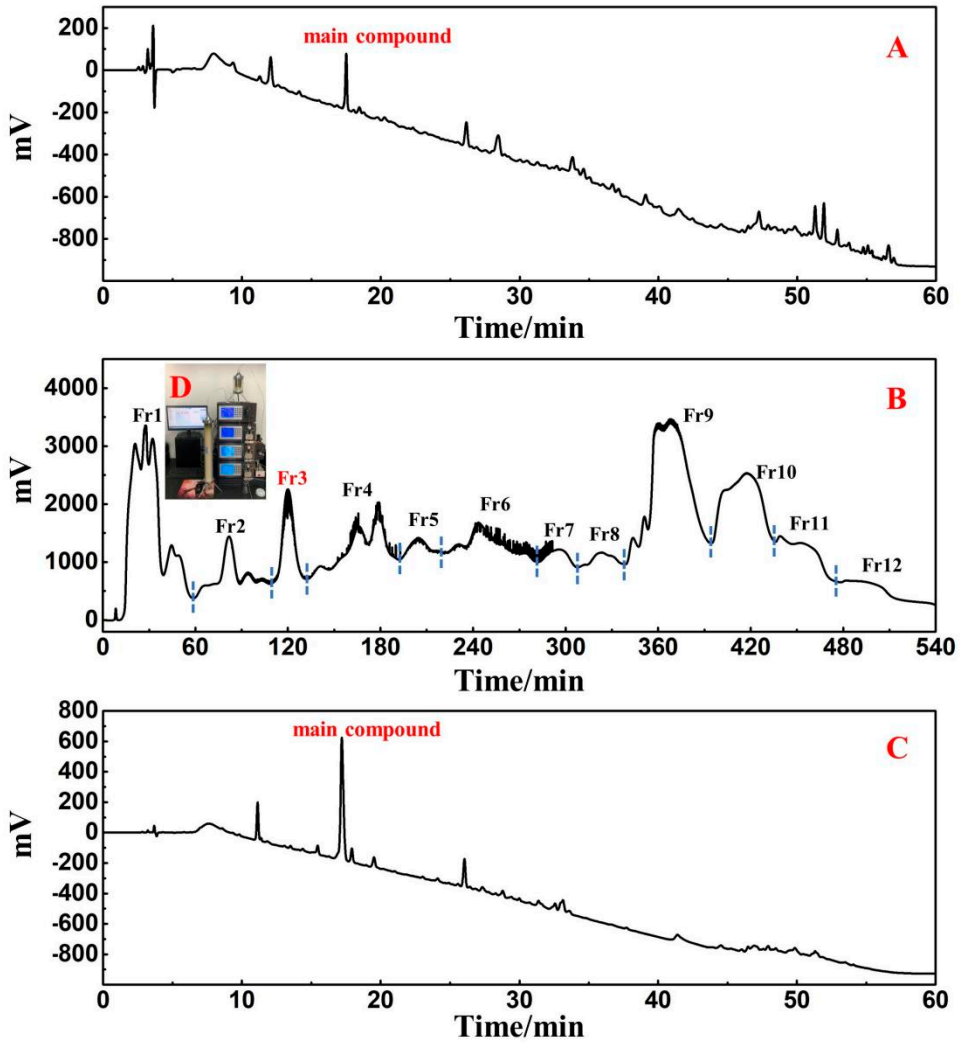

Figure 1. (**A**) The analytical chromatogram of crude Medicago sativa sample on the ReproSil−Pur C18 AQ analytical column. (**B**) The crude extract chromatogram of medium-pressure liquid chromatography pretreatment with MCI GEL® CHP20P as the stationary phase. (**C**) The analytical chromatogram of fraction Fr3 on the ReproSil-Pur C18 AQ analytical column. (**D**) The actual MCI GEL® CHP20P medium-pressure liquid chromatography system.

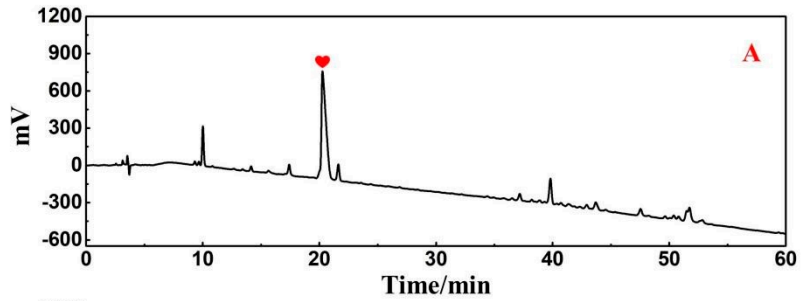

Figure 2. *Cont.*

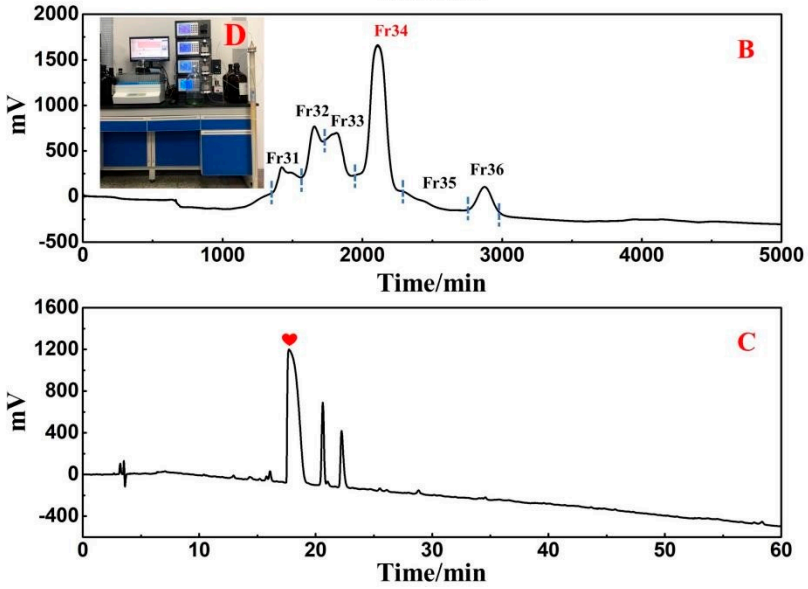

Figure 2. (**A**) The analytical chromatogram of fraction Fr3 on the ReproSil−Pur C18 AQ analytical column. (**B**) The fraction Fr3 chromatogram of medium-pressure liquid chromatography pretreatment with Sephadex LH-20 as the stationary phase. (**C**) The analytical chromatogram of fraction Fr34 on the ReproSil-Pur C18 AQ analytical column. (**D**) The actual Sephadex LH-20 medium-pressure liquid chromatography system.

3.2. High-Pressure Preparation and Purity Analysis of the Isolated Quality Marker

After optimizing the chromatographic conditions for the analysis of Fr34, the ReproSil-Pur C18 AQ analytical column was selected to further analyze Fr34 under the optimized chromatographic conditions. The resulting chromatogram is shown in Figure 3A. Thus, the subsequent preparation was more convenient and time-saving. The red-hearted peak in this chromatogram was the main compound's peak. Subsequently, the main compound was prepared on a high-pressure ReproSil-Pur C18 AQ preparative column with optimized chromatographic conditions for Fr34. The resulting chromatogram was Figure 3B. A sample of 63.4 mg of Fr341 (blue marked peaks) was prepared with a recovery of 17.6%. In order to clarify the purity of the target compound Fr341, the ReproSil-Pur C18 AQ analytical column was used to analyze Fr341. It could be seen from Figure 3C that the purity of the target compound is above 95%.

In order to confirm that the prepared Fr341 was the main compound in the crude extract, the ReproSil-Pur C18 AQ column was used to analyze and compare the crude extract and Fr341 under the same chromatographic conditions. The obtained chromatograms, Figure 4A,B, corresponded to the crude extract and Fr341, respectively.

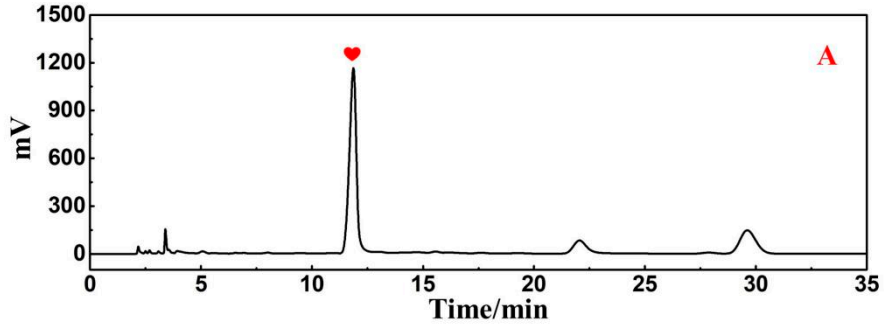

Figure 3. *Cont.*

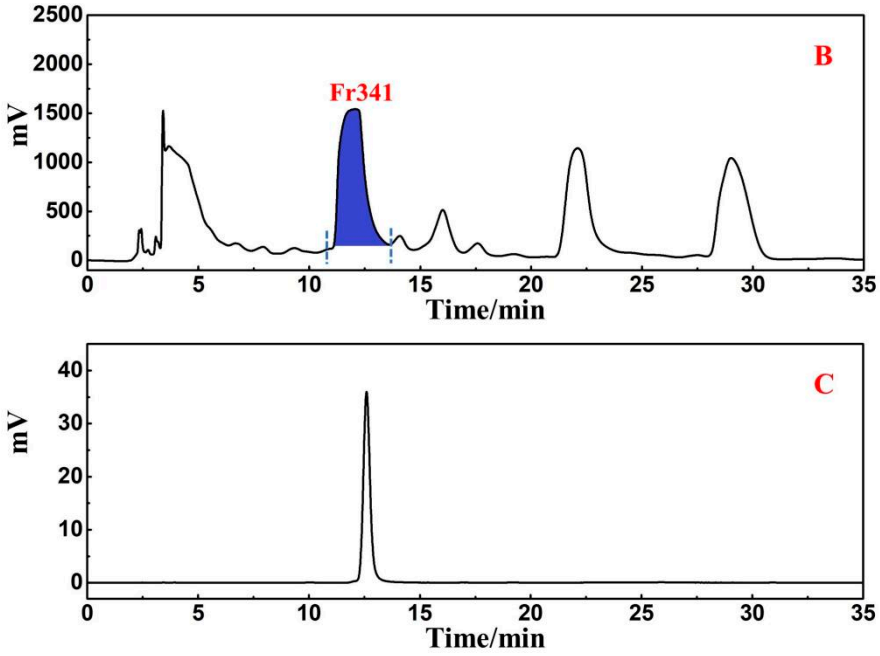

Figure 3. (**A**)The analytical chromatogram of fraction Fr34 on the ReproSil-Pur C18 AQ. (**B**) High-pressure preparative fraction Fr34 chromatogram using a ReproSil-Pur C18 AQ preparative column. (**C**) The analytical chromatogram of fraction Fr341 on the ReproSil-Pur C18 AQ.

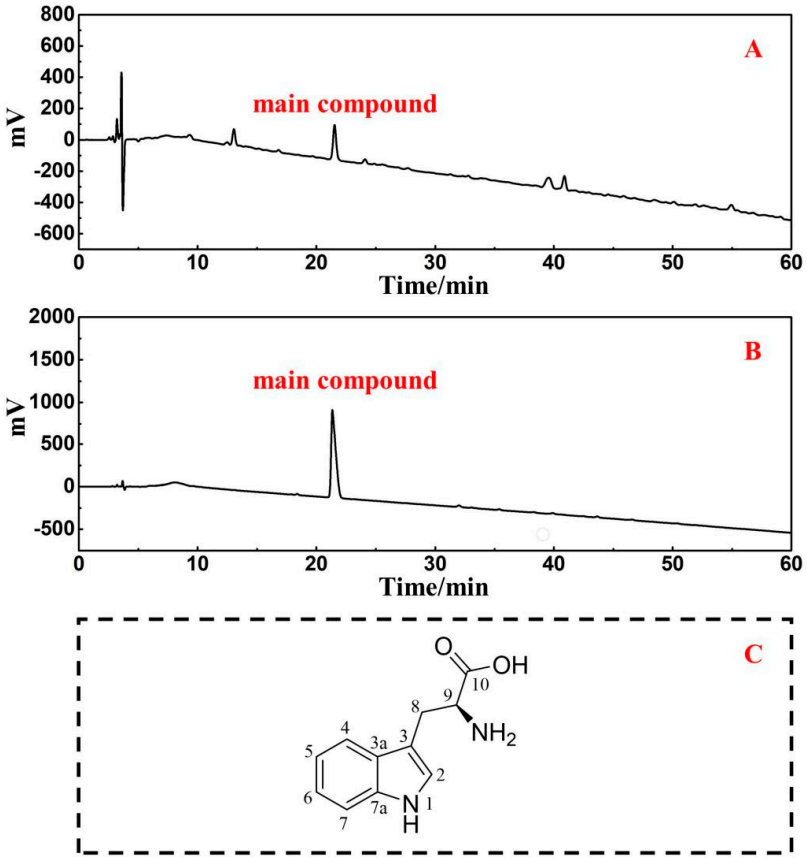

Figure 4. (**A**) The analytical chromatogram of fraction of crude Medicago sativa sample on the ReproSil-Pur C18 AQ analytical column. (**B**) The analytical chromatogram of fraction Fr341 on the ReproSil-Pur C18 AQ analytical column. (**C**) Structural formula of L-tryptophan.

3.3. Structural Characterization of the Isolated Quality Marker

In order to clarify the structure of Fr341, ESI-MS (Figure 5A,B), ^{1}H NMR (Figure 5C), and ^{13}C NMR (Figure 5D) spectra were obtained and matched with the published literature. According to all the spectral data, the target compound was L-tryptophan (see the list of NMR peaks and molecular ions below). The resulting L-tryptophan structural formula is shown in Figure 4C.

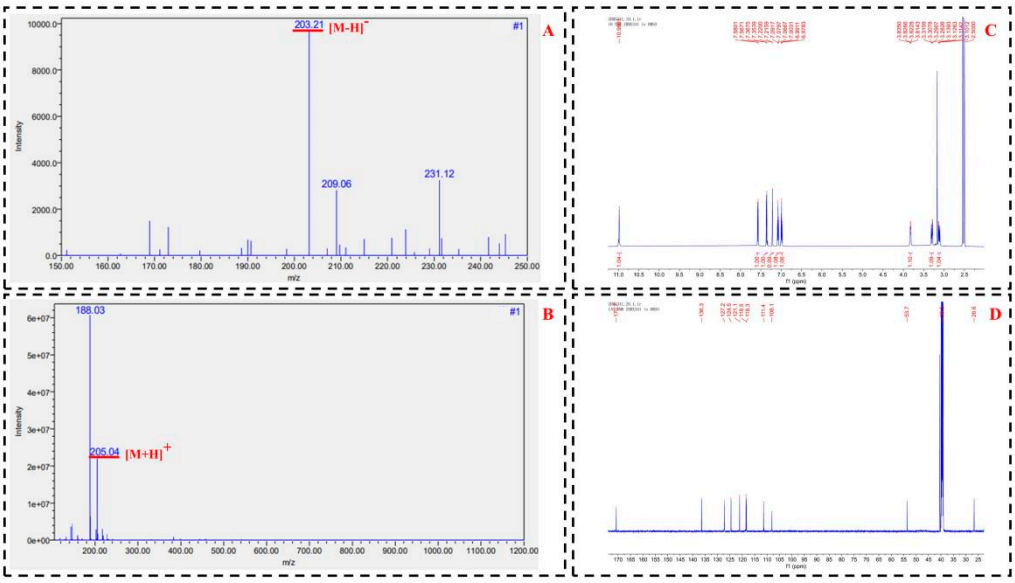

Figure 5. (**A**,**B**) Q−TOF−MS spectra of Fr341; (**C**) ^{1}H NMR spectra of Fr341; (**D**) ^{13}C NMR spectra of Fr341.

Fraction Fr341 (L-tryptophan, light-yellow powder, ESI-MS *m/z* 203.21, [M−H]$^{-}$: ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.98 (1H, d, NH-1), 7.57 (1H, d, *J* = 7.9 Hz, H-4), 7.36 (1H, d, *J* = 7.9 Hz, H-7), 7.22 (1H, d, *J* = 2.4 Hz, H-2), 7.08 (1H, t, *J* = 7.4 Hz, H-6), 6.99 (1H, t, *J* = 7.4 Hz, H-5), 3.82 (1H, dd, *J* = 7.3, 5.1 Hz, H-9), 3.30 (1H, dd, *J* = 15.1, 5.1 Hz, H-8a), 3.12 (1H, dd, *J* = 15.1, 7.3 Hz, H-8b); ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.7 (C-10), 136.3 (C-7a), 127.2 (C-3a), 124.5 (C-2), 121.1 (C-6), 118.5 (C-5), 118.3 (C-4), 111.4 (C-7), 108.1 (C-3), 53.7 (C-9), 26.6 (C-8). The data were in agreement with that of L-tryptophan in the literature [34].

4. Discussion

MeOH was chosen as the extraction solvent because of its high solubility and low cost. Dried *Medicago sativa* was cut into one-centimeter pieces to facilitate MeOH extraction of *Medicago sativa*. The purpose of mixing the concentrated extract obtained by rotary evaporation with silica gel was to consider the complexity and poor solubility of the extract. After mixing with silica gel and drying, it could be used for subsequent medium-pressure liquid chromatography pretreatment. The purpose of the MCI GEL® CHP20P pretreatment was to remove the chlorophyll in the methanol extract of *Medicago sativa* and at the same time achieve enrichment of the fraction. Because chlorophyll could be adsorbed on the preparative column's stationary phase, this would not contaminate the subsequent chromatographic columns we used. Because MCI GEL® CHP20P fillers were predominantly styrene–divinylbenzene copolymers, it was chosen as the stationary phase for this medium-pressure liquid chromatography procedure. This polymeric filler maintained a stable spherical structure in organic solvents, which could enable high reproducibility during purification of target compounds. This achieved high resolution and product recovery in this study. The analytical chromatogram in Figure 1C showed that with the same chromatographic conditions, the retention time of the main compound Fr3 was similar to

that of the crude sample, i.e., the major compound Fr3 was significantly enriched in the crude sample. Accordingly, we chose Fr3 as the target fraction for further separation.

Since the components in Fr3 were still relatively complex, to separate the peaks of the primary components, medium-pressure liquid chromatography was utilized. The stationary phase was Sephadex LH-20, because this stationary phase has been widely used because of its convenience and efficiency. It was a novel technique that facilitates the isolation of compounds from certain plant sources. Subsequently, the Fr34 obtained by the medium-pressure liquid chromatography in this step was analyzed using the same chromatographic conditions that were optimized to analyze Fr3. The resulting analytical chromatogram in Figure 2C was compared with Fr3 (Figure 2A). The comparison results showed that the retention time of the main compound Fr34 was similar to that of Fr3 (red heart marked peaks) with the same chromatographic conditions. This means that the main compound, Fr34, was significantly enriched in the crude sample. Accordingly, we chose Fr34 for the next high-pressure liquid chromatography preparation.

For more convenient preparation, the chromatographic conditions for analyzing Fr34 need to be further optimized before high-pressure liquid chromatography preparation. The final optimized chromatographic condition was 8% methanol isocratic. This isocratic chromatographic condition means that there was no need to set an equilibration time during the preparation to restore the system to the desired mobile phase ratio. Fr341 and crude extracts prepared under these chromatographic conditions were analyzed on a ReproSil-Pur C18 AQ column under the same chromatographic conditions. By comparing these two chromatograms (Figure 4A,B), it was found that the retention time of the Fr341 peak was similar to the peak time of the main compound in the crude extract. This proved that the prepared Fr341 was the main compound of alfalfa *Medicago sativa*. As a result, Fr341 can be used as the Q-marker of *Medicago sativa* for quality control.

These results could show that the combination of medium- and high-pressure chromatography can effectively separate tryptophan from *Medicago sativa*. In addition, this method can filter out other redundant components and focus on the key indicators (Q-markers) of quality control. The selected Q-marker has the best comprehensive characteristics of content and stability among the many complex components of the forage and can comprehensively characterize the intrinsic quality of the forage. Yang et al. provide a successful case of setting scientific quality standards based on Q-markers [13]. According to these cases, it was known that HPLC could be used to determine the characteristic components of traditional Chinese medicine and other products, which could also confirm the accuracy of our results based on MPLC and HPLC. Finally, this method enables large-scale separation and purification of reference materials from other forages.

5. Conclusions

In this study, medium-pressure liquid chromatography and high-pressure liquid chromatography were used to separate L-tryptophan. Medium-pressure liquid chromatography with MCI GEL® CHP20P as the stationary phase removed chlorophyll to enrich the target fraction. Sephadex LH-20 was used for further purification by isocratic methanol elution. After analysis and preparation by ReproSil-Pur C18 AQ, L-tryptophan was obtained, with a purity of more than 95%. From the conclusions, it can be shown that the method combined with medium- and high-pressure chromatography can separate and purify Q-markers from other forages on a large scale. In conclusion, the new method established in this study provides a valuable perspective on forage quality control. The method could be used for relevant commercial cultivation such as forage and feed and can isolate and purify reference compounds of quality markers for quality control from these commercial forages on a large scale.

Author Contributions: Conceptualization, J.D. and Q.W.; methodology, S.Z.; validation, S.Z., Y.M., and R.M.; investigation, S.Z.; resources, J.D.; writing—original draft preparation, R.M.; writing—review and editing, S.Z.; visualization, J.D.; supervision, Q.W.; project administration, Y.M.; funding acquisition, J.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are available upon request from the authors.

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

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