Optimization of headspace solid phase microextraction conditions and composition analysis of volatile constituents from *Trifolium pratense* L. leaves

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Abstract. Objective: To analyze the volatile constituents in the leaves of *Trifolium pratense* L. and to explore the effects of different factors on the extraction efficiency. Methods: The volatile components were extracted by the headspace solid phase microextraction (HS-SPME) method and analyzed by gas chromatography-mass spectrometry (GC-MS), and the relative content of the components was calculated by the area normalization method. Results: The best headspace solid phase microextraction conditions of the volatile components of the leaves of *Trifolium pratense* L. were as follows: 50 µm DVB/CAR/PDMS extraction head was used, the extraction temperature was 70 °C, and extraction time was 40min. 82 compounds were identified from *Trifolium pratense* L. leaves by GC-MS, accounting for 93.02% of the total volatile components, and the total peak area of volatile components was 2.31×10⁹. The compounds with higher content and active activity were beta-

Caryophyllene (6.48%) and Naphthalene, decahydro-4a-methyl-1-methylene-(1-methylene-7-(1-methylene-1-methylene) (4.11%). Conclusion: The type of extraction head, the extraction temperature and the extraction time have great influence on the extraction effect. The HS-SPME-GC-MS method is suitable for the rapid extraction, separation and analysis of the volatile components of the leaves of *Trifolium pratense* L.

1. Introduction

*Trifolium pratense* L. is a perennial herb of Trifolium in Leguminosae [1]. As a traditional folk herbal medicine, it is widely used in relieving cough, asthma and treating local ulcer [2]. It is widely planted in many provinces of China and has a large output. Trifolium pratense mainly contains saponins, flavonoids, coumarins and other active ingredients [3]. In recent years, it has been found that it has a very good effect in anti-osteoporosis, curing ulcerative colitis, improving cognitive disorders and other diseases [4-6].

A large number of Chinese herbal medicines containing volatile oil have been reported to have certain pharmacological activities in clinical treatment, such as antibacterial and anti-inflammatory, antipyretic and analgesic [7]. At present, there are few studies on the volatile components of Trifolium pratense. The essential oil of Trifolium pratense was extracted by steam distillation. And 81
compounds were identified by GC-MS [8]. Fat soluble components were extracted from Trifolium pratense seeds by Soxhlet extraction. Six fat soluble components were identified by GC-MS. [9] Our previous study found that the volatile components of Trifolium pratense from different areas are quite different. The number of compounds identified in the whole plant from Guizhou Province was the largest [10]. The volatile components in different parts of stems, leaves and flowers of Trifolium pratense in Guizhou are also quite different. There are the most abundant and unique components in the leaves, which may have great application value [11]. Therefore, in this experiment, leaves of Trifolium pratense from Guizhou Province were studied. By optimizing the collection conditions of HS-SPME and analyzing the composition of the volatile components in different optimization processes by GC-MS, the best extraction, separation and analysis methods of the volatile components in Trifolium pratense leaves were established. It played the certain roles of providing reference for the development and utilization of Trifolium pratense.

2. Materials and Apparatus

2.1. Plant materials

\textit{Trifolium pratense} L. was purchased from Guizhou Province and identified by Ouyang puyue, associate professor of Chinese Traditional Medicine Department, Guangdong food and Drug Vocational College. Leaves were selected as experimental materials. The certificate specimen is deposited in Guangdong food and Drug Vocational College.

2.2. Apparatus

The apparatus used in this study included: A11 pulverizer (aika (Guangzhou) instrument and Equipment Co., Ltd.); manual SPME injection handle (supelco company in the United States); 50 μ m DVB / car / PDMS extraction head, 65 μ m PDMS / DVB extraction head, 75 μ m car / PDMS extraction head, 85 μ m polyacrylate extraction head, 100 μ m PDMS extraction head (supelco company in the United States); 15 Ml headspace bottle (Ningbo HONGPU Instrument Technology Co., Ltd.); bsa202s electronic balance (saiduolis scientific instrument (Beijing) Co., Ltd.); gcms-qp2010 ultra gas chromatography-mass spectrometry (Shimadzu Manufacturing Institute, Japan).

3. Method

3.1. Steps of headspace solid phase microextraction

In this experiment, the extraction conditions of SPME were optimized, and the kinds of extraction heads, extraction temperature and extraction time were investigated. The steps are as follows: weigh 0.2g red clover leaves that have been crushed to fine powder, put them into a 15ml headspace bottle, use the extraction temperature to balance for 10min, insert the aged solid-phase microextraction fiber head (aging at 250 °C for 30min) for extraction, take them out after extraction for a certain time, and immediately insert them into the gas chromatograph sample inlet (250 °C) for analysis.

3.2. GC-MS Conditions

3.2.1. Gas chromatography conditions. The GC system used chromatographic separation was achieved by using a fused capillary column Rts-5MS, length (30m×0.25mm×0.25um). The initial column temperature was 50°C, and it was raised to 140 °C at 3°C/min for 5min. Then it was raised to 180 °C at 4°C/min for 10min, finally raised to 250 °C at 10°C/min. The carrier gas was high purity helium with a flow rate of 0.8 mL / min. The injection temperature is 250 °C, the interface temperature is 280 °C, the desorption temperature is 250 °C, and the desorption time is 5min. There is no split injection. The total time of GC is 62 min.
3.2.2. Mass spectrometry conditions. Electron bombardment ion (EI) source is used as ionization mode. The monitoring method is full scanning. The scanning range is 35-500. The ionization energy is 70eV. The ion source temperature is 230°C.

3.3. Qualitative analysis
Qualitative analysis was carried out by searching NIST 11 standard library, selecting compounds with a matching degree of more than 80%, and combining the results of literature reports for qualitative analysis. The relative percentage content of each compound in total volatile components was calculated by peak area normalization method.

4. Result

4.1. HS-SPME condition optimization

4.1.1. Selection of extraction head. At a temperature of 80°C, volatile components in Trifolium pratense leaves were extracted by different kinds of extraction head for 20 minutes, including 50 µm DVB / CAR / PDMS extraction head, 65 µm PDMS / DVB extraction head, 75 µm CAR / PDMS extraction head, 85 µm Polyacrylate extraction head, and 100 µm PDMS extraction head. The results in Figure 1 show that the 50 µm DVB / CAR / PDMS extraction head has good selectivity, and GC-MS can obtain the largest total peak area and chromatographic peak number, which are $1.93 \times 10^9$ and 70 peaks respectively. The smallest peak area was obtained by extraction with 100 µm PDMS extraction head. There was little difference in the number of chromatographic peaks obtained by 65 µm PDMS / DVB extraction head, 75 µm CAR / PDMS extraction head, 85 µm polyacrylate extraction head and 100 µm PDMS extraction head. Therefore, 50 µm DVB / CAR / PDMS extraction head is selected.

![Figure 1. Effect of different kinds of extractor on peak area and peak number of volatile components in leaves of Trifolium pratense L.](image)

4.1.2. Selection of extraction temperature. The effects of extraction temperature of 50, 60, 70, 80, 90 °C on the total peak area and peak number of volatile components in Trifolium pratense leaves were investigated. The purpose of solid phase microextraction is to rapidly enrich the volatile
components in *Trifolium pratense*. If the extraction temperature is low, the extraction of volatile components is incomplete. If the extraction temperature is high, and the extraction components have reacted. So it is very important to choose the appropriate extraction temperature. Fig. 2 shows that with the increasing of extraction temperature, the number of chromatographic peaks increases slowly and then decreases rapidly, and the total peak area keeps increasing. When the extraction temperature is $50\,^\circ\text{C}$, the minimum total peak area is $0.77 \times 10^9$, and when the extraction temperature is $90\,^\circ\text{C}$, the maximum total peak area is $3.09 \times 10^9$. At the extraction temperature of $90\,^\circ\text{C}$, the number of peaks obtained was at least 63. And at the extraction temperature of $70\,^\circ\text{C}$, the number of peaks obtained was at most 73. The higher the extraction temperature, the faster the volatilization rate of the volatile components in *Trifolium pratense* leaves. In the same time, the more the content of the substances absorbed by the extraction head, the more the total peak area detected by GC-MS. However, the increase of extraction temperature and the polymerization of volatile components lead to the decrease of chromatographic peak number. Therefore, $70\,^\circ\text{C}$ is used as the extraction temperature.

![Figure 2](image)

**Figure 2.** Effect of extraction temperature on peak area and peak number of volatile components in leaves of *Trifolium pratense*.

4.1.3. Selection of extraction time. Under the condition of extraction temperature of $70\,^\circ\text{C}$, $50\,\mu\text{m}$ DVB / CAR / PDMS extraction head was used to investigate the effect of extraction time of 20, 30, 40, 50, 60 min on the total peak area and the number of chromatographic peaks of volatile components in *Trifolium pratense* leaves. Fig. 3 shows that with the increase of extraction time, the number of chromatographic peaks increases slowly and then decreases rapidly, and the total peak area keeps increasing. When the extraction time is 20 minutes, the minimum total peak area is $1.58 \times 10^9$. When the extraction time is 60 minutes, the maximum total peak area is $2.78 \times 10^9$. When the extraction time was 20 minutes, the minimum number of chromatographic peaks was 73. When the extraction time was 40 minutes, the maximum chromatographic peaks was 83. The longer the extraction time is, the more the components are absorbed by the extraction head, and the more the total peak area is obtained by GC-MS. Therefore, 40 min is used as the extraction time.
4.2. Analysis results of volatile components in *Trifolium pratense*

The optimal extraction conditions were as follows: extraction temperature was 70 °C, extraction head was 50 μm DVB / CAR / PDMS extraction head, and extraction time was 40 min. According to the above optimization conditions, volatile components of *Trifolium pratense* leaves were obtained by GC-MS. From Figure 4 and table 1, 82 compounds were identified in *Trifolium pratense* leaves, accounting for 93.02% of the total volatile components, with a total peak area of $2.31 \times 10^9$. The compounds with higher content were Bicyclo[3.1.0]hexan-3-one, 4-methyl-1-(1-methylethyl) (10.13%), Eucalyptol (7.41%), β-caryophyllene (6.48%) and Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl) (4.11%).

**Table 1.** Species, peak area and relative content of volatile components in leaves of *Trifolium pratense* L.

| Serial number | tR/min | Compound name | Chemical formula | Peak area/10^8 | Percentage of compound in total area /% |
|---------------|--------|---------------|------------------|----------------|-----------------------------------------|
| 1             | 9.817  | Eucalyptol    | C_{10}H_{18}O     | 1.84           | 7.41                                    |
| 2             | 11.278 | 1,5-Heptadien-4-one, 3,3,6-trimethyl | C_{10}H_{16}O     | 0.24           | 0.96                                    |
| 3             | 11.876 | Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl) | C_{10}H_{18}O     | 0.29           | 1.18                                    |
| 4             | 12.461 | 1,5-Heptadien-4-ol, 3,3,6-trimethyl | C_{10}H_{18}O     | 0.10           | 0.4                                     |
| 5             | 13.74  | Bicyclo[3.1.0]hexan-3-one, 4-methyl-1-(1-methylethyl) | C_{10}H_{18}O     | 2.52           | 10.13                                   |
| 6             | 14.319 | Thujone       | C_{10}H_{16}O     | 0.51           | 2.05                                    |
| 7             | 14.735 | Hexanoic acid, 2-ethyl | C_{9}H_{16}O     | 0.02           | 0.09                                    |
| 8             | 15.482 | Bicyclo[3.1.0]hexan-3-ol, 4-methylene-1-(1-methylethyl) | C_{10}H_{18}O     | 0.08           | 0.31                                    |
| 9             | 16.165 | Dehydromevalonic lactone | C_{9}H_{14}O      | 0.13           | 0.52                                    |
| 10            | 16.52  | Pinocarvone   | C_{10}H_{18}O     | 0.06           | 0.25                                    |
| 11            | 16.81  | 2-Norpinanol, 3,6,6-trimethyl | C_{10}H_{18}O     | 0.33           | 1.32                                    |
| 12            | 17.066 | Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl | C_{10}H_{16}O     | 0.54           | 2.18                                    |
| 13            | 17.352 | Nonane, 3-methyl-5-propyl | C_{13}H_{28}      | 0.09           | 0.38                                    |
| No. | Retention Time (min) | Compound Name                                                                                   | Molecular Formula | Concentration (ppm) | Area (μA·s) |
|-----|----------------------|--------------------------------------------------------------------------------------------------|-------------------|---------------------|-------------|
| 14  | 17.503               | 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)                                                   | C10H18O           | 0.25                | 1           |
| 15  | 18.233               | alpha.-Terpineol                                                                                | C10H18O           | 0.38                | 1.53        |
| 16  | 18.821               | Decanal                                                                                         | C10H20O           | 0.18                | 0.72        |
| 17  | 19.25                | Undecane, 2,5-dimethyl                                                                          | C13H28            | 0.06                | 0.26        |
| 18  | 19.406               | 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)                                                 | C10H16O           | 0.08                | 0.32        |
| 19  | 19.662               | Dodecane, 4-methyl                                                                               | C13H28            | 0.04                | 0.15        |
| 20  | 21.584               | Tetradecane, 5-methyl                                                                           | C13H32            | 0.16                | 0.66        |
| 21  | 21.695               | 2-Cyclohexen-1-one,3-methyl-6-(1-methylethenyl)                                                 | C10H14O           | 0.07                | 0.28        |
| 22  | 22.183               | Nonane, 5-methyl-5-propyl                                                                         | C13H28            | 0.30                | 1.21        |
| 23  | 22.485               | Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-acetate                                              | C12H2O2           | 0.03                | 0.14        |
| 24  | 22.703               | Decane, 2,3,5-trimethyl                                                                          | C13H28            | 0.03                | 0.11        |
| 25  | 22.846               | Dodecane, 2-methyl                                                                               | C13H28            | 0.07                | 0.29        |
| 26  | 23.365               | Tridecane                                                                                       | C13H28            | 0.22                | 0.87        |
| 27  | 23.845               | 1,3-Cyclopentadiene, 5-(1,1-dimethylethyl)                                                     | C9H14             | 0.08                | 0.31        |
| 28  | 24.106               | 1-Octanol, 2-butyl                                                                               | C12H26O           | 0.11                | 0.45        |
| 29  | 24.328               | Dodecane, 4,6-dimethyl                                                                           | C14H30            | 0.26                | 1.04        |
| 30  | 24.882               | Pentadecane                                                                                     | C15H32            | 0.05                | 0.19        |
| 31  | 25.609               | Hexadecane                                                                                      | C16H34            | 0.11                | 0.45        |
| 32  | 25.965               | Heptadecane                                                                                     | C17H36            | 0.07                | 0.27        |
| 33  | 26.299               | Heptadecane                                                                                     | C17H36            | 0.20                | 0.79        |
| 34  | 26.623               | Copaene                                                                                         | C15H24            | 0.25                | 1           |
| 35  | 26.736               | Dodecane,2,7,10-trimethyl                                                                       | C15H32            | 0.15                | 0.6         |
| 36  | 26.962               | (+)-beta.-Bourbonene                                                                             | C15H24            | 0.23                | 0.92        |
| 37  | 27.233               | Cyclohexane,1-ethyliden-1-methyl-2,4-bis(1-methylethenyl)                                       | C15H24            | 0.52                | 2.1         |
| 38  | 27.826               | Pentadecane, 7-methyl                                                                           | C16H34            | 0.39                | 1.57        |
| 39  | 28.025               | Longifolene                                                                                     | C15H24            | 0.40                | 1.63        |
| 40  | 28.149               | 2,4,7,9-Tetramethyl-5-decyln-4,7-diol                                                            | C12H2O2           | 0.24                | 0.95        |
| 41  | 28.305               | Decane, 3,8-dimethyl                                                                             | C12H26            | 0.16                | 0.66        |
| 42  | 28.518               | beta.-Caryophyllene                                                                              | C15H24            | 1.61                | 6.48        |
| 43  | 28.695               | Tetradecane, 4-methyl                                                                            | C15H32            | 0.09                | 0.38        |
| 44  | 28.944               | 1H-Cyclopenta[1,3]cycloprop[1,2]benzene, octahydro-7-methyl-4-(1-methylethyl)                   | C15H24            | 0.18                | 0.73        |
| 45  | 29.128               | Bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl)                                    | C15H24            | 0.16                | 0.63        |
| 46  | 29.302               | Altoaromadendrene                                                                               | C15H24            | 0.41                | 1.65        |
| 47  | 29.715               | 5,9-Undecadien-2-one, 6,10-dimethyl                                                             | C15H32            | 0.13                | 0.51        |
| 48  | 29.994               | Cyclohexane-1-methanol, 3,9-dimethyl-2-(3-methyl-1,3-butadienyl)                                 | C15H24            | 0.90                | 3.62        |
| 49  | 30.386               | Octadecane                                                                                      | C18H38            | 0.21                | 0.86        |
| 50  | 30.889               | gamma.-Muurolene                                                                                | C15H24            | 0.48                | 1.95        |
| 51  | 31.147               | alpha.-Cubebene                                                                                 | C15H24            | 0.56                | 2.27        |
| 52  | 31.513               | Naphthalene, decahydro-4a-methyl-1-methylene-1-(1-methylethyl)                                 | C15H24            | 1.02                | 4.11        |
| 53  | 31.865               | Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethyl)                          | C15H24            | 0.68                | 2.72        |
| 54  | 32.222               | Butylated Hydroxytoluene                                                                         | C15H32O           | 0.54                | 2.19        |
| 55  | 32.504               | Phenol, 2,4-bis(1,1-dimethylethyl)                                                               | C11H2O            | 0.31                | 1.24        |
5. Discussion

82 kinds of volatile compounds were identified in *Trifolium pratense* leaves, among which 27 compounds with relative content ≥ 1%, accounting for 68.22% of the total compounds. *Trifolium pratense*, as a traditional medicine, has a wide range of medicinal value. However, there is no in-depth study on the volatile components and their efficacy. The volatile components of *Fructus aurantii*, lavender, cinnamon and other Chinese herbal medicines have the effects of promoting gastrointestinal motility, bacteriostasis, improving memory disorders and so on [12-14]. The volatile components of *Trifolium pratense* may also have certain efficacy. According to the results of analysis and literature review, it was found that the highest relative content of 4-methyl-1 - (1-methylethyl) bicyclo [3.1.0] hexane-3-one had no report on its pharmacological effect. The other volatile components identified have a variety of physiological...
activities. Eucalyptol and Copaene have cardiovascular pharmacological activity[15]. β-caryophyllene can alleviate the brain injury induced by cerebral ischemia-reperfusion injury in mice[16]. β-caryophyllene, Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl), (-)-Globulol and Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl) are the main components of the essential oil of the lotus leaves. The results of MTT test showed that the essential oil of the lotus leaves significantly inhibited the proliferation of human lung cancer cells A549 and H460 [17]. Gamma.-Murolene is the main component of the essential oil from the roots of Panax quinquefolium, which had good effect on anti-inflammatory, antipyretic and analgesic, and inhibit fungi [18].

The results showed that the leaves of Trifolium pratense were rich in medicinal volatile components, which had great development value. In this study, HS-SPME technology was optimized to extract the volatile components from Trifolium pratense leaves, and GC-MS was used to analyze the components, providing a scientific reference for further research. Therefore, in the follow-up study, we can further explore the pharmacological effect and mechanism of the volatile components in Trifolium pratense leaves. This experiment lay the foundation for the application of Trifolium resources.

Acknowledgments
This work was financially supported by Young Innovative Talents Project of General colleges and universities in Guangdong Province (2019GKQNCX064) and Natural Science Research Project of Guangdong Food and Drug Vocational College (2019ZR15).

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