Potency Analysis of Twelve Cannabinoids in Industrial Hemp via UPLC-MS/MS

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Abstract

This study validated an UPLC-MS/MS analysis method to simultaneously detect twelve cannabinoids. The concentration-response relationship for all analyzed cannabinoids was linear with $R^2$ values > 0.99 using the developed method, the relative standard deviations of recovery for the three levels of spiked samples are between 66.1%~104.1%. The method was used to analyze 43 industrial hemp flower and leaf samples, with the data being statistically analyzed. The analysis results showed that the cannabinoids content varied significantly among different varieties and different pretreatment methods, sample heating treatment led to the decarboxylation of acidic cannabinoids and the mutual conversion of cannabinoids. Based on the statistical analysis of the cannabinoids, hemp from different regions and different varieties were well distinguished by the PLS-DA model, with the main contributing substances being Cannabidiol, $\Delta^9$-tetrahydrocannabinol, and $\Delta^8$-tetrahydrocannabinol.

1.Introduction

In China, Industrial hemp (Hemp) is defined as a cannabis plant of the genus Cannabis sativa L. that has been cultivated based on traditional Chinese strains with a $\Delta^9$-tetrahydrocannabinol (Δ⁹-THC) content of less than 0.3% Wt in the dried flowers and leaves¹. There are various strains for fiber, seed, and flower/leaf extraction for medicinal use. Many provinces and regions in China have traditional hemp cultivation, mostly for hemp seeds consumption. Heilongjiang province is mainly cultivated in fiber hemp, only Yunnan province have issued local regulations permitting for the cultivation and processing of industrial hemp for medical use.

Cannabinoids are terpenophenolic compounds unique to cannabis, primarily synthesized from the precursor cannabigerolic acid (CBGA) generated via two pathways: the fatty acid pathway and the terpene pathway, with CBGA being the primary precursor for cannabinoid biosynthesis catalyzed by the enzyme cannabigerolic (CBG) synthase. CBGA is subsequently converted into cannabidiolic acid (CBD) or tetrahydrocannabinolic acid (THCA-A) by CBG synthase and THCA-A synthase, respectively. CBGA, CBD, and THCA-A are then decarboxylated to yield the final products CBG, CBD, and THC, respectively². Notably, the single THCA-A synthase or CBDA synthase can synthesize both THC and CBD simultaneously, with THCA synthase chiefly producing THC while synthesizing trace amounts of CBD, and CBD synthase chiefly producing CBD while synthesizing trace amounts of THC. THC and CBD are isomers of each other and are often present in high and low or nearly equal amounts depending on the homozygous or heterozygous status of the co-dominant gene B locus, making them the two most abundant cannabinoids in cannabis plants³. Under storage or heat conditions, acidic cannabinoid components can undergo decarboxylation reactions⁴-⁵.

CBD and THC have significant pharmacological activity and can bind to CB₁ and CB₂ receptors to exert their unique pharmacological effects⁶. Studies indicate that CBD has high medicinal value in treating depression⁷, anxiety⁸, epilepsy⁹, cardiovascular and cerebrovascular diseases¹⁰, respiratory diseases¹¹-¹², and other conditions. Although THC is an addictive psychoactive substance, it has excellent prospects in
pain relief, anti-inflammatory\cite{13}, anticonvulsant \cite{14}, anti-allergic\cite{15}, appetite stimulation, and Alzheimer’s disease treatment. The mutual conversion and synergistic effects between cannabinoids are also the main trend in current research on cannabinoid drug applications\cite{16}. In recent years, other cannabinoid components such as CBG and cannabinol (CBN) have also garnered increasing attention in the medical field due to their unique pharmacological effects\cite{17-19}. Ultra-high performance liquid chromatography-tandem mass spectrometry\cite{20} boasts numerous advantages, including high accuracy, wide analytical range, good selectivity, and high sensitivity\cite{21}. Thus, based on the UHPLC-MS/MS method, this experiment comprehensively detected 12 cannabinoids with a detection time of only 11 minutes and optimized the sample pretreatment method and validated the analytical method, providing an effective analytical technique for the regulation of China’s industrial hemp industry.

2. Materials and Methods

2.1 Experimental Instruments and Materials

2.1.1 Instruments

The liquid chromatography-tandem quadrupole mass spectrometer with electrospray ionization (ESI) used was the Waters ACQUITY UPLC Xevo TQD, equipped with a Waters Masslynx 4.1 data processing system, both manufactured by Waters Corporation, USA. The ultrapure water machine was supplied by Millipore Corporation, USA, and the electronic balance was the Sartorius BP211D, from Sartorius Scientific Instruments Co., Ltd., Germany. The ultrasonic extractor was the KQ-300DA CNC, from Shanghai Bolong Electronic Co., Ltd., and the handheld high-speed grinder was the DFT-200, from Wenzhou Lindam Machinery Co., Ltd. The air blast drying oven was the GZX-9140 MBE, from Shanghai Boxun Industrial Co., Ltd.

2.1.2 Materials

The reference standards for cannabidivarin (CBDV, batch number: FE05121901, purity: 99.9%), cannabidiolic acid (CBDA, batch number: FE04301903, purity: 99.5%), cannabinoids (CBG, batch number: FE05181901, purity: 99.6%), cannabidiol (CBD, batch number: FE10071912, purity: 99.8%), tetrahydrocannabivarin (THCV, batch number: FE10111901, purity: 99.5%), $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, batch number: FE01041701, purity: 98.1%), $\Delta^8$-tetrahydrocannabinol ($\Delta^8$-THC, batch number: FE12271903, purity: 99.7%), cannabichromene (CBC, batch number: FE10281904, purity: 99.2%), and tetrahydrocannabinolic acid A (THCA-A, batch number: FE09271901, purity: 98.12%), internal standard (IS) Cannabidiol-D3 (CBD-D3, batch number: FE12121902, purity: 99.6%), were purchased from Cerilliant, Sigma-Aldrich, USA. Cannabigerolic acid (CBGA, batch number: FE01082007, purity: 99.5%), cannabinol (CBN, batch number: FE01020013, purity: 99.6%), and cannabicyclol (CBL, batch number: FE01212010, purity: 99.1%), were purchased from Supelco, USA. Ultrapure water was prepared using the Milli-Q system, Millipore, USA. Acetonitrile, formic acid, isopropanol, methanol, and other reagents for sample pretreatment were all analytical or chromatography grade. The reagents for LC-MS were all mass spectrometry grade.

Industrial hemp flower and leaf samples were grown and collected in different regions of Yunnan Province from 2020 to 2022, and some were provided by Yunnan University and Yunnan Hemp Industry Association. These samples were categorized into four groups: local varieties Yunma (Yunma No.7, Yunma No.8, Yunma No.10) (NO.1˜24), foreign introduced varieties (NO.25˜38), Chinese research varieties (NO.39˜42), and unknown illegal planted variety (NO.43) (Table S1).

2.2 Preparation of Standard Solution

12-cannabinoid mixed standard solution with a concentration of 40 $\mu$g/mL was prepared by diluting 1 mL of 1000 $\mu$g/mL standard solutions of CBDV, CBDA, CBGA, CBG, CBD, THCV, CBN, $\Delta^9$-THC, $\Delta^8$-THC, CBC, THCA-A, and CBL, respectively, with methanol to a 25 mL volumetric flask. The solution was stored at -18°C for no more than one month. When in use, the 12-cannabinoids mixed standard solution of 40 $\mu$
g/mL was diluted with methanol to prepare a series of working solutions with concentrations of 0.01, 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL for the establishment of a standard curve, and 2 µg/mL internal standard (IS) CBD-D3 was added in each mixed standard solution at the same time.

2.3 Preparation of sample

2.3.1 Pretreatment of sample

Sample of hemp flowers and leaves: About 10 g of sample with a moisture content of less than 10% was taken in a grinder, and 0.1 g (accurate to 0.0001 g) of the crushed sample was weighed into a 10 mL volumetric flask, 7-8 mL of 95% methanol aqueous solution was added for extraction, sonicated for 30 min, then dilute to volume 10 mL, and 2 µg/mL IS CBD-D3 was added in each sample solution at the same time.

2.3.2 Preparation of sample solution for recovery

This sample preparation and extraction was performed as described in a paper by Tran et al. (Tran et al., 2022) Recovery samples were prepared by spiking 20 mg of sample with 10 µL of 100 µg/mL standard and then prepared to 1 mL in 95% methanol. A 1:10 dilution of the extract was performed to achieve a concentration of 0.1 µg/mL. The medium spike (MS) was prepared by adding 20 µL of 100 µg/mL standard and followed the steps mentioned previously to achieve concentrations of 0.2 µg/mL. The high spike (HS) was prepared by adding 200 µL of 100 µg/mL standard and followed the steps mentioned previously to achieve concentrations of 2 µg/mL. Samples were sonicated and transferred into 2 mL amber HPLC vials. A further 1 in 2 dilution was required to ensure all cannabinoids fit within the calibration curve, making the total dilution 1 in 20. Final spikes concentrations were 0.05 µg/mL (low spike, LS), 0.1 µg/mL (medium spike, MS), and 1 µg/mL (high spike, HS).

2.4 Experimental Conditions

2.4.1 Liquid Chromatography

The chromatographic column used was the Waters Acquity BEH-C18 (2.1 mm×50 mm, 1.7 µm). The mobile phase was a 0.1% formic acid aqueous solution (A)-acetonitrile (B) gradient elution: 0˜3.80 min, 70% B; 3.81˜7.20 min, 77% B; 7.21˜9.00 min, 90% B; 9.01˜11.00 min, 100% B, with a flow rate of 0.3 mL/min. The column temperature was 30 , and the injection volume was 1µL.

2.4.2 Mass Spectrometry

The electrospray ionization source was used, with ESI positive and negative ion modes scanned simultaneously. The capillary voltage was 2.68 kV, and the desolvation gas temperature was 650. The desolvation gas flow rate was 850 L/h, and the cone gas flow rate was 50 L/h. The collision gas was argon, and the MRM multiple reaction monitoring mode was used. After optimization of mass spectrometry conditions, the retention time, molecular ion, characteristic fragment ion, and collection parameters of each substance are shown in the table below (Table 1).

<table>
<thead>
<tr>
<th>NO.</th>
<th>RT (min)</th>
<th>Compound Name</th>
<th>Ion Mode</th>
<th>Precursor Ion (m/z)</th>
<th>Quantificational/Qualitative ion (m/z)</th>
<th>Cone (V)</th>
<th>Collision (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.44</td>
<td>CBDV</td>
<td>P</td>
<td>287.12</td>
<td>165.03*/92.96 34</td>
<td>22/32</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.38</td>
<td>CBDA</td>
<td>P</td>
<td>359.18</td>
<td>341.20*/219.06 20</td>
<td>16/28</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.75</td>
<td>CBG</td>
<td>P</td>
<td>317.19</td>
<td>193.24*/123.12 22</td>
<td>22/34</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.91</td>
<td>CBD</td>
<td>P</td>
<td>315.17</td>
<td>193.04*/92.95 34</td>
<td>22/34</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.38</td>
<td>THCV</td>
<td>P</td>
<td>287.12</td>
<td>165.03*/92.96 32</td>
<td>24/22</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.74</td>
<td>CBN</td>
<td>P</td>
<td>311.14</td>
<td>223.03*/241.14 46</td>
<td>30/20</td>
<td></td>
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<tr>
<td>7</td>
<td>6.77</td>
<td>Δ⁹-THC</td>
<td>P</td>
<td>315.17</td>
<td>193.04*/92.95 34</td>
<td>22/34</td>
<td></td>
</tr>
<tr>
<td>NO.</td>
<td>RT (min)</td>
<td>Compound Name</td>
<td>Ion Mode</td>
<td>Precursor Ion (m/z)</td>
<td>Quantificational/Qualitative ion (m/z)</td>
<td>Cone (V)</td>
<td>Collision (V)</td>
</tr>
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<tr>
<td>8</td>
<td>6.95</td>
<td>Δ8-THC</td>
<td>P</td>
<td>315.17</td>
<td>193.04⁴/92.95 34</td>
<td>22/34</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7.74</td>
<td>CBL</td>
<td>P</td>
<td>315.28</td>
<td>235.28⁷/81.18 32</td>
<td>16/30</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.29</td>
<td>CBC</td>
<td>P</td>
<td>315.17</td>
<td>193.04⁴/92.95 34</td>
<td>22/34</td>
<td></td>
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<tr>
<td>11</td>
<td>8.50</td>
<td>THCA-A</td>
<td>P</td>
<td>359.18</td>
<td>341.20⁷/219.06 20</td>
<td>16/28</td>
<td></td>
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<tr>
<td>12</td>
<td>3.71</td>
<td>CBGA</td>
<td>N</td>
<td>359.33</td>
<td>315.39⁹/341.41 42</td>
<td>18/20</td>
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</tr>
<tr>
<td>13</td>
<td>3.91</td>
<td>CBD-D3</td>
<td>P</td>
<td>318.17</td>
<td>196.20⁷/43.51 30</td>
<td>30/36</td>
<td></td>
</tr>
</tbody>
</table>

* quantifier ion

### 3. Method Validation

#### 3.1 Optimization of the Method

##### 3.1.1 Extraction Solvent

The efficacy of different solvents, including n-hexane:ethyl acetate (9:1, 7:3, 1:1, 3:7, 1:9), pure methanol, 95% methanol-water, and acetonitrile, were compared for their extraction efficiency of cannabinoids. It was found that the n-hexane:ethyl acetate system elicited unwanted interference with cannabinoids detection, including a large solvent peak and unstable compound retention time. The extraction rates of pure methanol and acetonitrile were marginally lower than that of 95% methanol-water, which was ultimately selected as the optimal extraction solvent.

##### 3.1.2 Extraction Method

The extraction efficiency of the No. 10 flower and leaf samples was evaluated under three different conditions: static, ultrasonic, and vortex. Under static conditions, the contents of CBD, THCA-A, CBDV, and CBG changed with time, slowly increasing within 10 hours and doubling the initial content after 24 hours. The cannabis components may have undergone transformation, prompting the need for prompt sample analysis. Ultrasonic extraction achieved the highest extraction rate in approximately 30 minutes. As prolonged ultrasonic time can cause an increase in temperature and decarboxylation of acidic cannabinoids, 30 minutes was deemed optimal. Vortex extraction demonstrated similar efficiency to ultrasonic extraction, but ultrasonic operation was simpler. Therefore, ultrasonic extraction for 30 minutes was chosen. (Table S3)

#### 3.2 Reproducibility and Precision

Accuracy and precision of the method was assessed by calculating the mean result of seven injections and determining the percent relative standard deviation (%RSD) of the repeat injections. This was conducted relevant experiments on three levels of standard samples and two random samples. Repeated injections of the 0.25, 1 and 5 µg/mL standards resulted in a %RSD < 9.09 for all analytes that are within the linear range of the method. Mean cannabinoid content and %RSD for two random strains was determined with values ranging between 2.48 and 6.04%. For the above mixed standards and two random strains, three consecutive injections were made every two hours for a total of 24 hours, and the peak area response values were recorded. The RSD range of each peak area was 4.27% to 11.91%. The daily precision was measured every day, with three consecutive measurements taken every day for five days, and the peak area response values were recorded. The RSD range was 5.26% to 10.99%, indicating good daily precision.

#### 3.3 Linearity

The calibration curve was prepared from 10 concentration gradients of methanol working standard solutions. The detection limit (LOD) and quantification limit (LOQ) were approximately 0.05 to 4.32 ng/mL and 0.17 to 14.38 ng/mL, respectively. The R² values of each cannabis component were 0.9990 or higher (Table 2). The LC-MS Chromatogram of 12 Cannabinoids as follows (Fig. 1).
Table 2. Linear equation, $R^2$, concentration range, LOD and LOQ for each cannabinoid.

<table>
<thead>
<tr>
<th>Cannabinoid</th>
<th>Calibration curve</th>
<th>$R^2$</th>
<th>Concentration (ng/mL)</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBDV</td>
<td>$Y=86001X-117.18$</td>
<td>0.9996</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>1.27</td>
<td>4.19</td>
</tr>
<tr>
<td>CBDA</td>
<td>$Y=53359.1X+49.73$</td>
<td>0.9996</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>0.72</td>
<td>2.40</td>
</tr>
<tr>
<td>CBG</td>
<td>$Y=121298X-218.6$</td>
<td>0.9990</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>2.69</td>
<td>8.95</td>
</tr>
<tr>
<td>CBD</td>
<td>$Y=120743X-118.41$</td>
<td>0.9997</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>2.03</td>
<td>6.77</td>
</tr>
<tr>
<td>THCV</td>
<td>$Y=42358.6X-34.28$</td>
<td>0.9991</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>2.29</td>
<td>7.63</td>
</tr>
<tr>
<td>CBN</td>
<td>$Y=86802.7X+30.88$</td>
<td>0.9999</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>1.26</td>
<td>4.19</td>
</tr>
<tr>
<td>$\Delta^9$-TH$^+$</td>
<td>$Y=71029.9X-47.50$</td>
<td>0.9994</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>4.32</td>
<td>14.38</td>
</tr>
<tr>
<td>$\Delta^8$-TH$^+$</td>
<td>$Y=43225.6X-48.38$</td>
<td>0.9991</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>2.22</td>
<td>6.67</td>
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<tr>
<td>CBL</td>
<td>$Y=125266X-338.77$</td>
<td>0.9998</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>1.55</td>
<td>5.16</td>
</tr>
<tr>
<td>CBC</td>
<td>$Y=33479.9X+20.94$</td>
<td>0.9997</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>2.03</td>
<td>6.76</td>
</tr>
<tr>
<td>THCA-A</td>
<td>$Y=259993X+22.85$</td>
<td>0.9996</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>0.60</td>
<td>2.00</td>
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<td>CBGA</td>
<td>$Y=8925.5X-31.10$</td>
<td>0.9997</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>0.05</td>
<td>0.17</td>
</tr>
<tr>
<td>CBD-D$_3$</td>
<td>$Y=90041X-120.52$</td>
<td>0.9998</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;-500</td>
<td>0.02</td>
<td>0.07</td>
</tr>
</tbody>
</table>

3.4 Recovery Investigation

Six consecutive injections of the test sample solution were performed, and the recovery rate was calculated using the following formula:

$$\text{Recovery rate} = \frac{C_1 - C_0}{V_1} \times 100\%$$

where:
- $C_1$ is the measured concentration after adding standard, unit: $\mu$g/mL
- $C_0$ is the measured concentration of standard reserve solution of mixed control before adding standard, unit: $\mu$g/mL
- $V_1$ is the volume of standard added, unit: mL
- $M$ is the mass of standard added, unit: $\mu$g

The recovery rate results demonstrate that, when the mixed standard is added at a concentration of 0.05 $\mu$g/mL, the average recovery rate ranged from 84.53% to 99.88%, with a relative standard deviation (RSD) of 1.97% to 4.60%. Similarly, when the mixed standard is added at a concentration of 0.1 $\mu$g/mL, the average recovery rate ranged from 85.04% to 117.98%, with an RSD value of 0.97% to 4.48%. Moreover, when the mixed standard is added at a concentration of 1 $\mu$g/mL, the average recovery rate ranged from 75.69% to 109.88%, with an RSD value of 2.13% to 6.06%. Notably, the high, medium, and low concentration levels all achieved the targeted recovery rate. (Table S5)

3.5 Investigation of Matrix Effect

The peak area of cannabinoids in the samples was recorded, and the matrix effect was calculated. The formula for calculating the matrix effect is as follows: Matrix Effect (%) = (Peak area of matrix-matched standard solution) / (Peak area of reference solution) × 100%. When the concentrations of 12 cannabinoids in industrial hemp samples were 0.05 $u$g/mL, 0.1 $u$g/mL, and 1 $u$g/mL, the matrix effects were 86.82%−105.26%, 87.62%−109.84%, and 70.42%−110.75%, respectively. (Table S5)
4. Results and Discussion

4.1 Test Results of UPLC-MS/MS

Through the established UPLC-MS/MS analysis method, 43 industrial hemp samples harvested in Yunnan were subjected to analysis. Using Metabo Analyst 5.0 online data analysis platform, a multivariate principal component analysis was carried out on the cannabinoid chemical components of these four groups of sample.

4.1.1 Temperature Investigation

Increasing temperature can cause the conversion of cannabinoids, particularly the conversion of acidic cannabinoids to neutral cannabinoids. This part of study investigated three heated temperatures for the flower and leaf samples by oven drying, 105, 130, and 150, and for 10, 20, 30, 40, 50 and 60 minutes at each temperature, then quantified the content of the 12 cannabinoids. The result showed that after heating at 105 for 1 hour, the detected content of Δ^9-THC, Δ^8-THC, THCV and CBC was higher than the highest content at other temperatures (Fig.2A). CBD appeared to have a significant inflection point at approximately 30 minutes under 130 heating conditions, with the CBD content significantly higher than the other two groups of experiments, and then the CBD content decreased significantly, indicating that time control was critical (Fig.2B). All cannabinoids, except for the three acidic cannabinoids (CBDA, THCA-A, and CBGA), as well as CBN, reached their peak values under 150 and 50 minutes (Fig.2C). In order to convert the acidic cannabinoids in the sample to neutral cannabinoids as much as possible and obtain a stable CBD content, the following comparative experiment we used 130 and 30 minutes as sample pretreatment condition. (Table S4)

Note: The graph’s vertical coordinates on the left side represent the contents of Δ^9-THC, CBD, CBDA, Δ^8-THC, CBDV, and CBC, while the vertical coordinates on the right side represent THCA-A, THCV, CBG, CBGA, CBN, and CBL.

4.1.2 The Comparison of 12 Cannabinoids in Heated and Unheated Samples (Table S2)

From the overall data of all samples, both heated and unheated, it was observed that the content of CBDV, CBD, CBN, Δ^8-THC, Δ^3-THC, CBC, and CBG increased significantly after oven heating. Conversely, the content of CBDA, CBGA, and THCA-A decreased significantly after heating, which can be attributed to the thermal decarboxylation of acidic cannabinoids. In the unheated samples, the order of cannabinoid content from high to low is: CBDA, CBD, Δ^8-THC, Δ^3-THC, CBDV, CBC, CBN, THCA-A, CBGA, CBG, THCV, CBL. In the heated samples, the order of cannabinoid content is: CBD, Δ^8-THC, Δ^3-THC, CBDV, CBN, CBC, THCV, CBG, CBGA, THCA-A, CBL. This pattern is depicted in the violin plot (Fig. 3A).

The transformation law of Δ^9-THC and Δ^8-THC is consistent, and the increase and decrease caused by temperature change are consistent. As can be seen from the correlation diagram (Fig. 3C), there is a strong positive correlation between Δ^9-THC and Δ^8-THC in the same sample. The content of Δ^9-THC and Δ^8-THC in foreign introduced varieties decreased slightly after heating, while the content of Δ^9-THC and Δ^8-THC in Yunnan local varieties increased significantly after heating (about 1-3 times higher than before heating). The increase or decrease in Δ^9-THC and Δ^8-THC before and after heating always maintains the same frequency, but the increase or decrease of Δ^8-THC is slightly higher than that of Δ^9-THC. For all the samples we detected, whether heated or unheated, the content of Δ^8-THC were higher than that of Δ^9-THC. Δ^8-THC is currently discovered to be a psychoactive substance with an efficacy equivalent to about two-thirds of Δ^9-THC[24]. This results of analysis might indicate some new challenges to the regulation of the industrial hemp industry[25-26]. THCA-A will be converted to THC after decarboxylation by heating. From the data, more than 90% of THCA-A decreases after heating, but the total increase in Δ^8-THC and Δ^9-THC after heating is much greater than the decrease of THCA-A. Therefore, the increase in the content of Δ^8-THC and Δ^9-THC after heating is not only from the decarboxylation of THCA-A but may also come from the conversion of other cannabinoids[27]. The content of THCV in local varieties of samples increased by more than double after heating, which might be related to the high content of its precursor THCV. However, this trend was not found in foreign introduced varieties, and more than half of foreign introduction
varieties showed a decrease in THCV content after heating.

After heating pretreatment, the CBD content of all 43 industrial hemp samples was found to rank first among all tested cannabinoids. Related studies suggested that CBD may convert to THC under heating conditions[28]. The CBDV content increased significantly after heating and could double or more compared to the unheated sample. The CBGA content of all samples decreased over 90% after heating, and the increase of CBG of foreign introduction samples was lower than that of Yunnan domestic samples. The CBN content of both domestic and foreign introduction varieties increased after heating, but foreign samples increasing more than domestic samples. As CBN is the main metabolite of THC and has shown good activity in pharmacological research[29], the combination of THC and CBN can synergistically enhance neuroprotective effects[30]. The CBC content increased also after heating, CBL did not change much before and after heating and was the least among the 12 cannabinoids found in all the samples.

A PLS-DA (Fig. 3B) was created based on our cannabinoid content data. Principal components 1 and 2 contributed 33.5% and 44.4%. The heating pretreatment had a great influence on the cannabinoid content, and there was a clear difference between the heated and unheated samples on the PLS-DA diagram.

4.2 Principal component analysis of different hemp varieties

The top six cannabinoids in Yunma hemp varieties were CBDA, CBD, Δ⁸-THC, Δ⁹-THC, THCA-A, and CBDV, while which were CBD, CBDA, Δ⁸-THC, Δ⁹-THC, CBC, and CBN in the foreign-introduced variety samples. CBD of foreign-introduced varieties was the highest cannabinoid, about 2-3 times that of local industrial hemp samples at least. The CBD content of Chinese research varieties we detected was around 1.5% with Δ⁹-THC less than 0.3%, but foreign samples with high CBD content also had Δ⁹-THC content over 0.3%. Sample No.43 was an unknown variety of illegal planted confiscated by the government anti-drug department. The types and contents of cannabinoids in No.43 were less than those of regularly planted varieties, but the THCA-A content was around ten times than that of Yunma varieties, so that the Δ⁹-THC content was far higher than 0.3% under heating conditions, this might bring safety hazard. Therefore, industrial hemp planting in China must be legal seeds variety, standardized planting and storage, and strictly supervised by the government anti-drug department to ensure that the psychotropic substance Δ⁹-THC is within control.

The PLS-DA (Fig. 4A) of the four types of flower and leaf samples showed that principal components 1 and 2 both contributed 24.5%, and the four types of samples were clearly distinguished, especially the irregularly planted varieties, which were significantly different from the other three types of samples. Based on the VIP score (Fig. 4B), THCA-A, CBGA, CBG and CBDV had the greatest impact on this difference (VIP scores > 1), which was consistent with the experimental results.

5. Conclusion

In this paper, we have established a UPLC-MS/MS method for the simultaneous and rapid quantitative determination of twelve cannabinoids in industrial hemp flowers and leaves in 11 minutes. We have optimized the sample pre-treatment method, 95% methanol aqueous solution was selected as the extraction solvent, ultrasonic extraction for 30 minutes was used. Through method validation, the concentration-response relationship for all analyzed cannabinoids were linear with R² values > 0.99, the relative standard deviations of recovery for the three levels of spiked samples are between 66.1%~104.1%. Using the established method, we have analyzed 43 samples of industrial hemp flowers and leaves from 4 categories. We have compared the cannabinoid substances difference after thermal conversion, and the differences in cannabinoid content between different varieties. Based on the statistical analysis of the cannabinoids, hemp from different regions and different varieties were well distinguished by the PLS-DA model. The establishment of our analytical method and the analysis results of our batch data provide technical support and basic data accumulation for the research of cannabinoid components in different industrial hemp producing areas and varieties around the world.

Funding
Data availability
Data will be made available on request.

Declaration of Competing 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.

Supplementary materials
See supplementary file (Tables S1- S5, Fig.1S).

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