Application Report from Shimadzu

- **Mass Spectrometry**

LCMS-QTOF-004

Analysis and identification of phenylethanoid glycosides in cistanche tubulosa with LC and quadropole TOF-MS

Abstract: In this study, a rapid and accurate method was established for the identification of phenylethanoid glycosides in the aqueous extract of cistanche tubulosa by Shimadzu Liquid Chromatography-quadrupole time-of flight mass spectrometry (LCMS-9030). The liquid chromatography separation conditions and mass spectrometric detection conditions were established for the aqueous extract of cistanche tubulosa. First, the reference substances echinacoside and acteoside were analyzed and their secondary fragmentation patterns were summarized. Second, the major chromatographic peaks were identified using the Formula Predictor and ACD/Labs software, based on the obtained primary and secondary high-resolution mass spectrometry data of the components, by comparison with the fragmentation characteristics of the reference substances and references; a total of 18 compounds were identified. The results showed that the application of Shimadzu Liquid Chromatography-quadrupole time-of flight mass spectrometry with high resolution and accuracy could improve the efficiency of chemical composition analysis of traditional Chinese medicine and facilitate the discovery and identification of compounds.

Key words: Liquid Chromatography-quadrupole time-of flight mass spectrometry; cistanche tubulosa; aqueous extract; phenylethanoid glycosides

Herba Cistanche is the dry succulent stem, bearing scaly leaves, of Cistanche of Orobanchaceae. In China, herba cistanche is mainly found in the northwestern desert areas, such as Inner Mongolia and Xinjiang. Because of its excellent medicinal value, it is known as "desert ginseng". There are 6 species of herba cistanche recorded in China's higher plant key. After further investigation by Tu Pengfei and other domestic scholars, it was determined that there were in fact 4 species and 1 variety: cistanche deserticola, cistanche tubulosa, cistanche salsa, cistanche salsa with white flower, and cistanche sinensis. Herba cistanche finds application in kidney-replenishing, benefiting menstrual blood, and relaxing bowel. It is included among the top-quality medicine in the book of *Sheng Nong's Herbal Classic*: "With sweet, mild and non-toxic taste, it can cure various diseases and pathogenic factors, strengthen the middle warmer, remove the penis pains, maintain five internal organs, nourish yin, benefit the vital essence and keep young after long administration."

There are many components in cistanche tubulosa, such as phenylethanoid glycosides, iridoids, monoglycosides, lignans, and polysaccharides. Among them, the phenylethanoid glycosides are the main characteristic component of cistanche tubulosa. Many researchers have shown that the main index component of herba

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cistanche is echinacoside, which has broad and significant pharmacological applications and has significantly higher content in cistanche tubulosa than in other species of herba cistanche. Moreover, due to its low price, it can be considered for practical applications and its clinical value is enhanced.

In this paper, Shimadzu high-resolution LCMS-9030 was used, with its high quality and accuracy, in combination with the Formula Predictor and ACD/Labs software, to efficiently and accurately identify the relevant components. This work was of great significance in terms of enriching the research content on the chemical composition of cistanche tubulosa, summarizing the mass fragmentation patterns of related compounds, and performing the quality evaluation, development, and utilization of medicinal materials.

1. Experiments

1.1 Apparatus

Shimadzu UPLC Nexera system and quadrupole time-of-flight mass spectrometer LCMS-9030 were used. The Nexera system included an LC-30AD×2 pump, a DGU-20A5 online degasser, a SIL-30AC auto-sampler, a CTO-20AC column oven, an SPD-M20A diode array detector, and a CBM-20A system controller. Data acquisition and analysis were performed on a LabSolutions Ver5.95 workstation. Mass spectra analysis was performed on a ACD/Labs Ver2012 software.

1.2 Conditions of Analysis

LC conditions

Column: Inertsil ODS-4 2.1 mm I.D. ×150 mm L, 5 μm

Mobile phase: Phase A: 0.1% formic acid aqueous solution; Phase B: methanol

Flow rate: 0.5 mL/min

Column temperature: 40 °C

Injection Volume: 5 μL

Detection wavelength: 190−800 nm

Elution mode: Gradient elution with initial concentration of mobile phase B being 5% (see Table 1 for the time program).

Table 1 Gradient elution program

Time(min)	Module	Command	Value
40.0	Pumps	Pump B Conc.	37
43.0	Pumps	Pump B Conc.	60
46.0	Pumps	Pump B Conc.	60
46.1	Pumps	Pump B Conc.	5
50.0	Controller	Stop	

MS Conditions

Ionization mode: ESI(-)
Heating gas: air, 10.0 L/min
Nebulizing gas: Nitrogen, 3.0 L/min
Drying gas: Nitrogen, 10.0 L/min
Collision gas: Argon
Interface temperature: 300 °C
DL temperature: 250 °C
Heater block temperature: 400 °C
Scan mode: MS full scan m/z: 100-1000
MSMS (DDA) m/z: 100-500 CE: 40±10 V
Loop time: 0.2 s

2. Sample pretreatment

Echinacoside (5.2 mg) and acteoside (6.1 mg) were weighed, dissolved in methanol to a volume of 10 mL to prepare stock solutions of concentrations 0.52 mg/mL and 0.61 mg/mL, respectively, diluted with methanol by 100 times to obtain test solutions of concentrations 5.2 mg/L and 6.1 mg/L, respectively, before loading onto the system for assay.

Water was added to the solid sample at the solid-liquid ratio 1:15, extracted for 2 h at 80 °C, cooled, and centrifuged. The supernatant was obtained, filtered through an ultrafiltration membrane (30,000u) to remove macromolecular components, and the permeate was concentrated four times by nanofiltration before loading onto the system for assay.

3. Results and Discussion

3.1 Study on the secondary mass spectrometry rules of reference substances

Echinacoside and acteoside are the main components in cistanche tubulosa. The reference substances prepared as described above were analyzed under the conditions described in section 1.2. The phenylethanoid glycosides have good mass spectral response in the negative ion mode and generate strong [M-H]- quasi-molecular ion peaks. Therefore, the results of negative-ion-mode spectroscopy were selected for analysis. The mass number accuracy of echinacoside and acteoside was less than 1 ppm, as shown in Table 2. Figure 1 shows the UV-vis chromatogram of echinacoside and acteoside. Figure 2 shows the ion-extraction flow diagrams for echinacoside and acteoside. The structures of the high-abundance fragments in the secondary high-resolution mass spectrum were analyzed and the possible fragmentation patterns were deduced using the ACD/Labs software. Figures 3 and 4 show the possible secondary fragmentation patterns of echinacoside and acteoside.

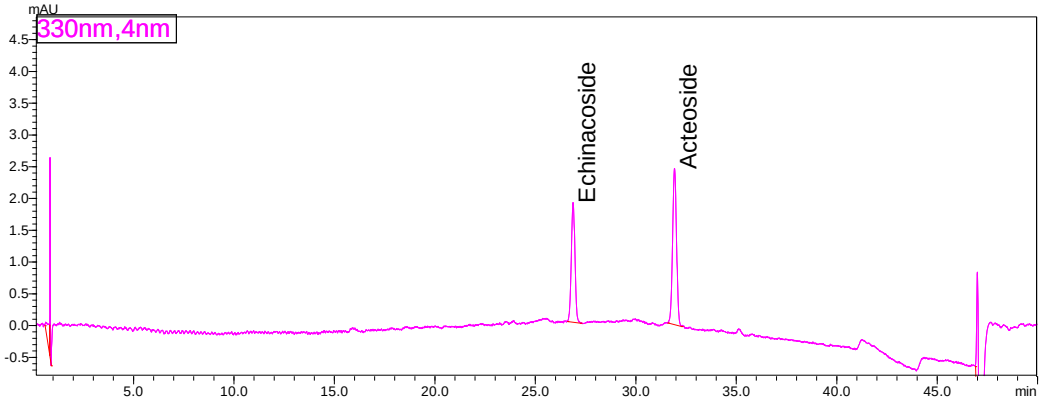


Figure 1. UV-vis chromatogram of echinacoside and acteoside

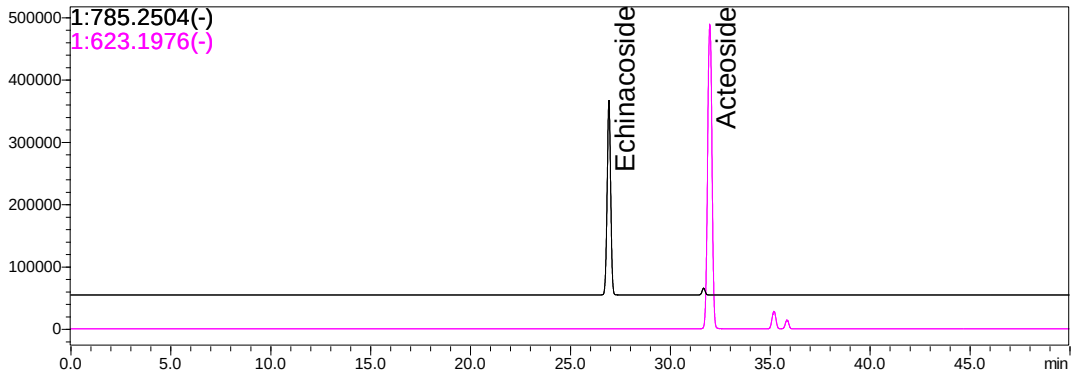


Figure 2. Ion-extraction flow diagrams for echinacoside and acteoside

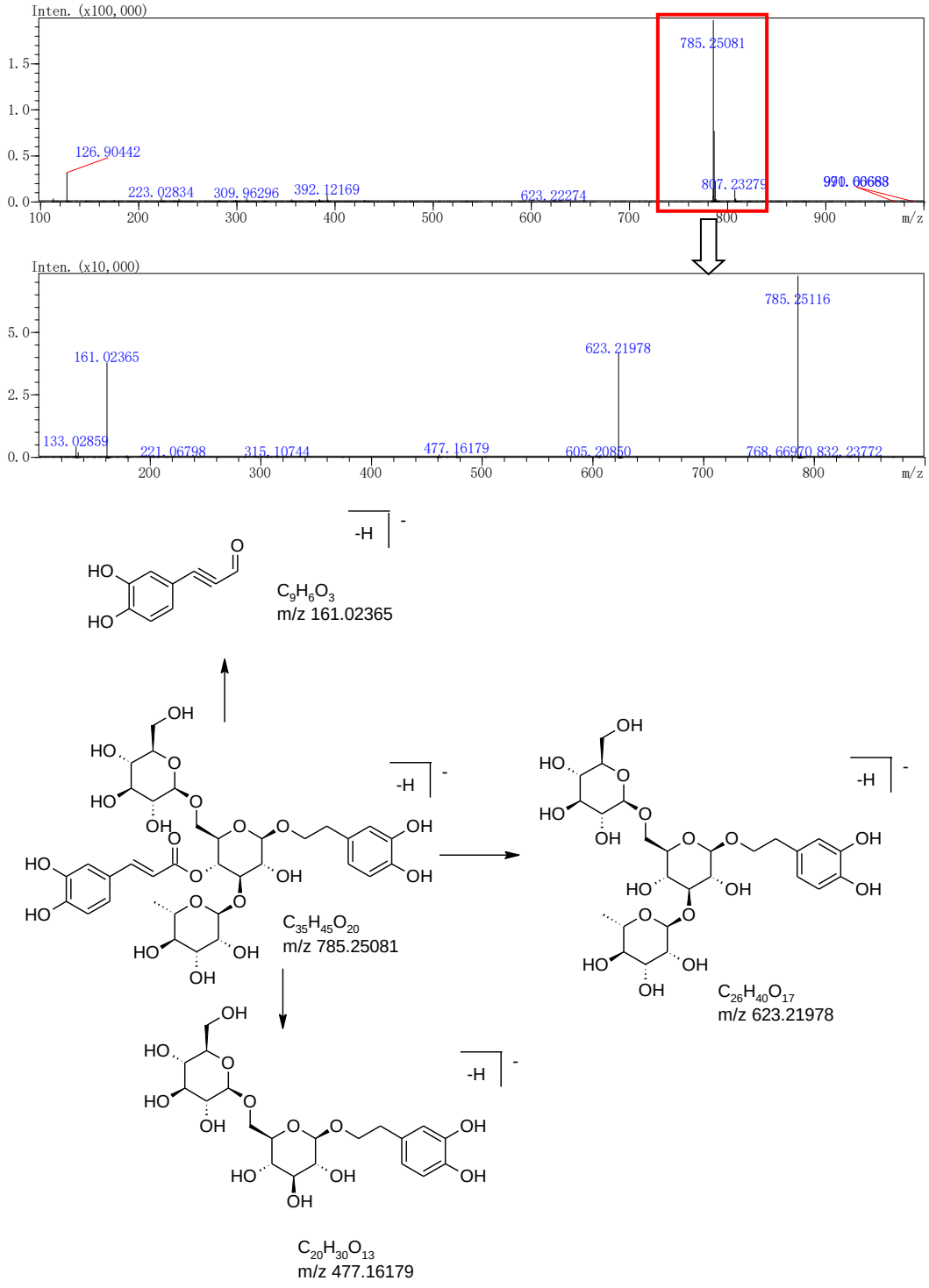
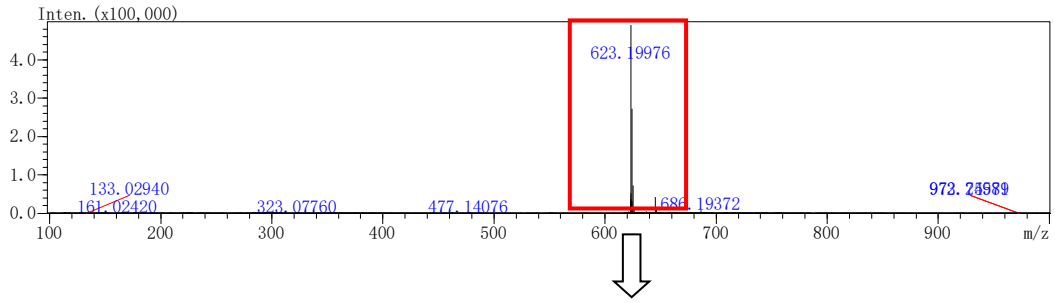


Figure 3. Possible secondary fragmentation patterns of m/z 785.25081



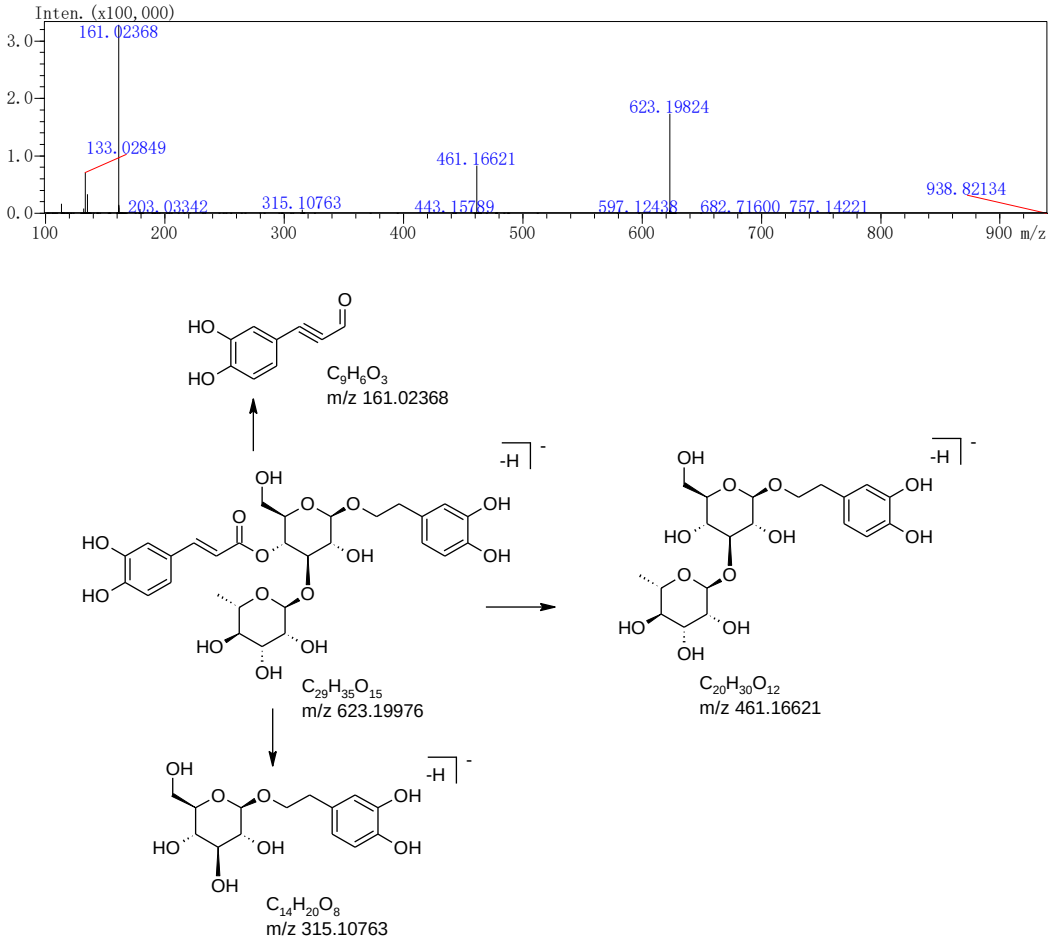


Figure 4. Possible secondary fragmentation patterns of m/z 623.19976

Table 2. Summary of chromatography-mass spectrometry data of the reference substances

No.	Compound name	Retention time/min	Molecular Formula	Theoretical m/z	Actual m/z	Mass deviation/ppm	Fragment m/z
1	Echinacoside	26.870	$C_{35}H_{46}O_{20}$	785.25042	785.25081	0.50	623.21978，161.02365，477.16179
2	Acteoside	32.012	$C_{29}H_{36}O_{15}$	623.19760	623.19815	0.89	161.02368，461.16621，315.10763

3.2 Study on phenylethanoid glycosides in cistanche tubulosa

The aqueous extract samples of cistanche tubulosa were diluted 100 times and analyzed under the conditions described in section 1.2. Figure 5 shows the UV chromatogram of the samples and Figure 6 shows the total ion chromatogram (TIC) obtained in the negative ion mode. According to literature reports and the properties of phenylethanoid glycosides, the elemental composition was set to C, H, and O, with the maximum values being 150, 300, and 12, respectively. Based on the high-resolution mass spectrometry data, Formula Predictor software was used to predict the possible molecular formula. The structures of the high-abundance fragments in the secondary high-resolution mass spectra were analyzed and the possible fragmentation patterns were deduced using ACD/Labs, to further confirm the molecular formula and structure of the compounds. Figures 8 and 9 show the possible secondary fragmentation patterns of tubuloside A and decaffeoylacteoside, respectively, with

high response.

In conclusion, a total of 18 chemical components were identified using the Formula Predictor and ACD/Labs software, compared with the fragmentation patterns of the reference compounds, along with the UV data, retention time, secondary mass spectrometry information, and references, as shown in Table 3.

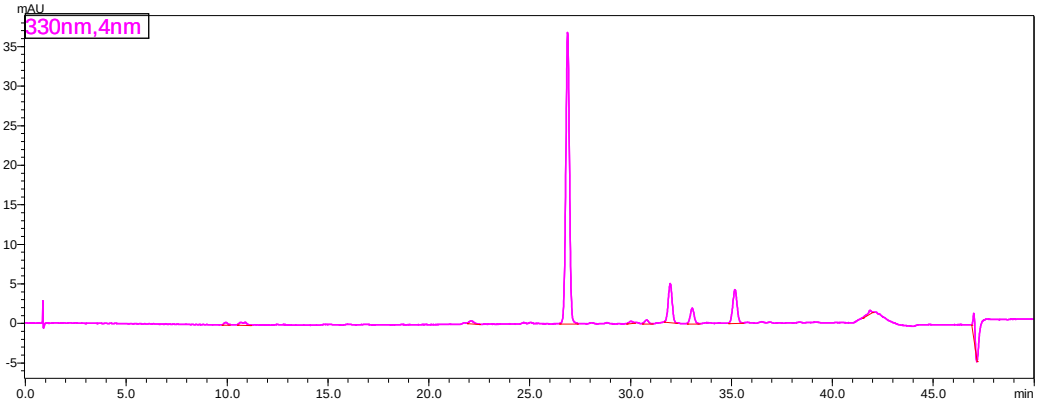


Figure 5. UV chromatogram of the samples

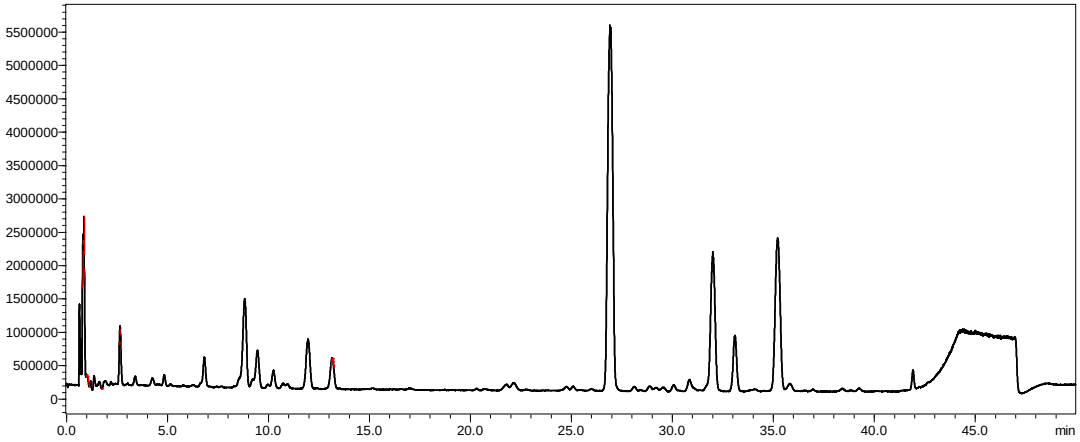


Figure 6. TIC of the samples

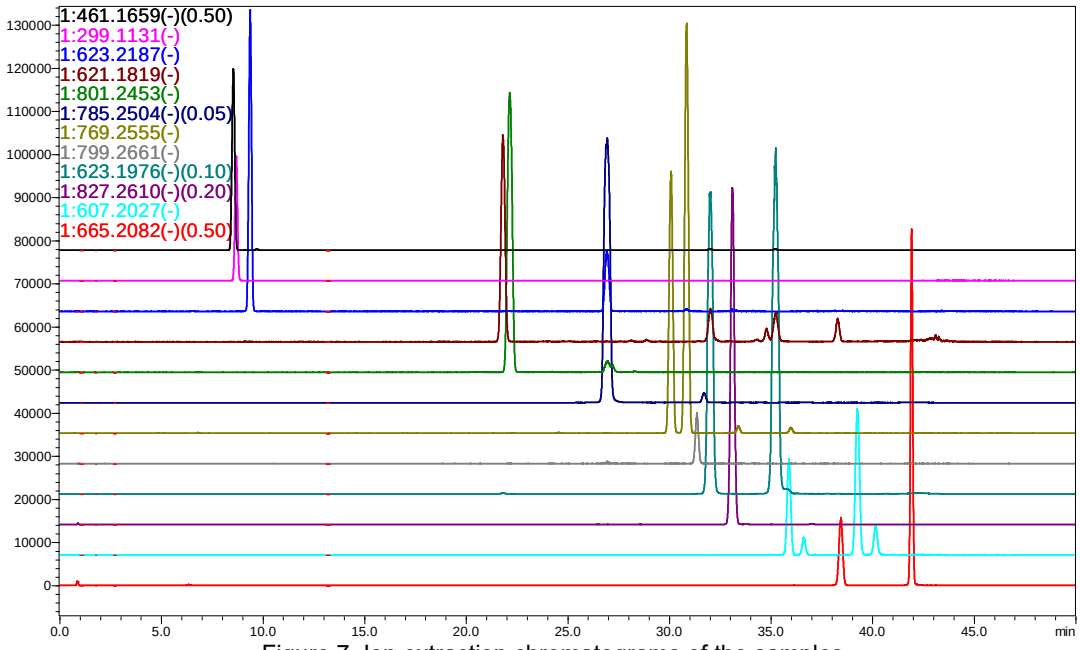


Figure 7. Ion extraction chromatograms of the samples

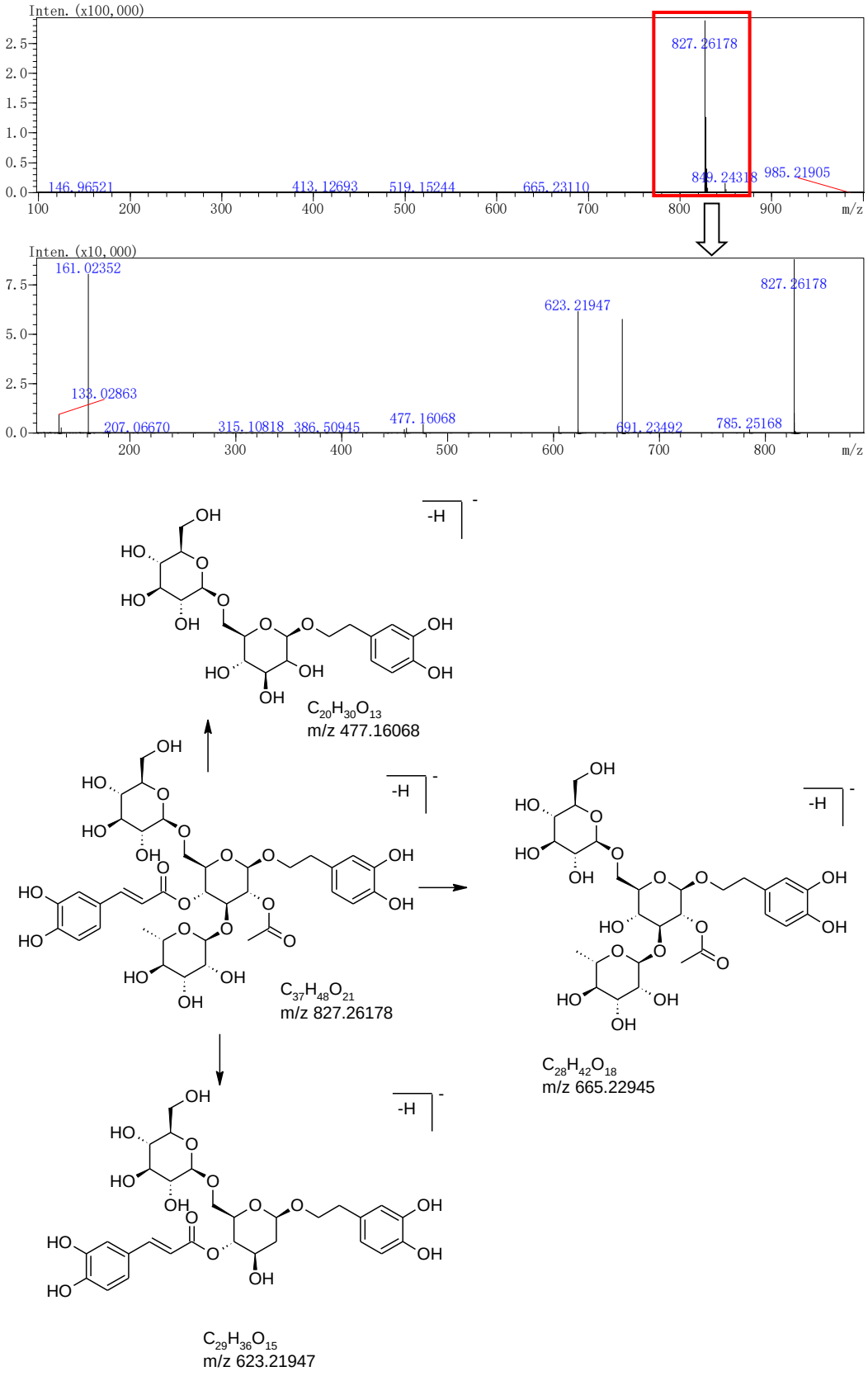


Figure 8. Possible secondary fragmentation patterns of m/z 827.26274

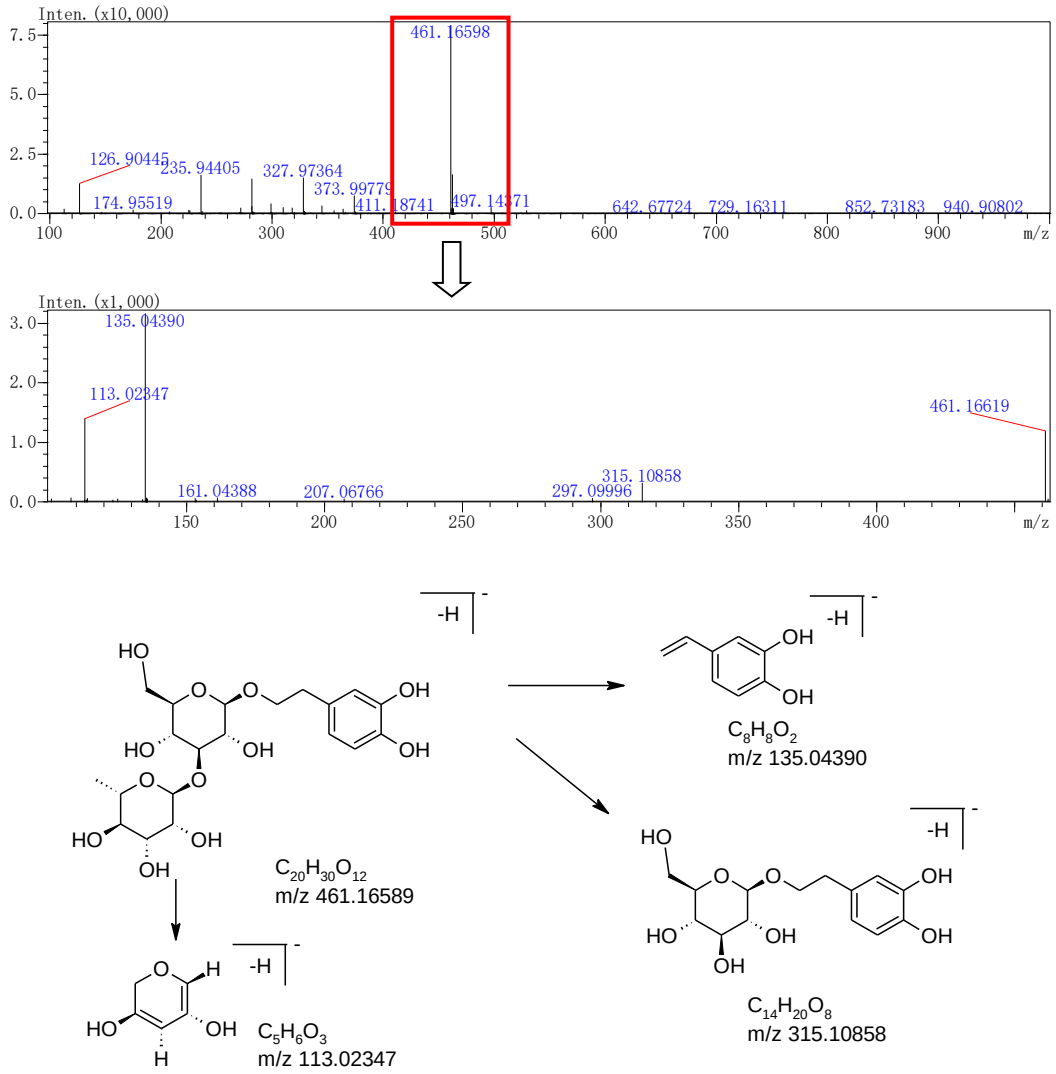


Figure 9. Possible secondary fragmentation patterns of m/z 461.16589

Table 3. Results based on which dentification of phenylethanoid glycosides in cistanche tubulosa was performed

No.	Retention time/min	Molecular Formula	Theoretical m/z	Actual m/z	Mass deviation/ppm	Fragment m/z	Identification results
1	8.550	$C_{20}H_{30}O_{12}$	461.16590	461.16598	0.17	135.04390, 113.02347, 315.10858	Decaffeoylacteoside
2	8.663	$C_{14}H_{20}O_7$	299.11308	299.11324	0.54	207.00919, 119.05041, 126.90484	Salidroside
3	9.301	$C_{26}H_{40}O_{17}$	623.21873	623.21908	0.57	125.02582, 221.07904, 135.04506	Kankanoside F
4	21.812	$C_{29}H_{34}O_{15}$	621.18195	621.18258	1.02	475.12499, 269.08111, 295.06034	Crenatoside

5	22.150	$C_{35}H_{46}O_{21}$	801.24533	801.24562	0.36	161.02342, 621.20417, 783.23661	Cistantubuloside C1/C2
6	26.870	$C_{35}H_{46}O_{20}$	785.25042	785.25081	0.50	623.21846, 161.02332, 477.16193	Echinacoside
7	30.085	$C_{35}H_{46}O_{19}$	769.25551	769.25563	0.16	161.02358, 607.23112, 133.02833	Cistantubuloside A
8	30.880	$C_{35}H_{46}O_{19}$	769.25551	769.25585	0.45	623.21917, 145.02844, 605.20870	Cistantubuloside B1/ B2
9	31.382	$C_{36}H_{48}O_{20}$	799.26607	799.26580	0.34	623.22103, 175.03950, 477.18801	Cistanoside A/Wiedemanninoside C
10	32.012	$C_{29}H_{36}O_{15}$	623.19760	623.19815	0.89	161.02353, 461.16639, 315.10849	Acteoside
11	33.128	$C_{37}H_{48}O_{21}$	827.26098	827.26178	0.96	665.22945, 623.21947, 477.16068	Tubuloside A
12	35.241	$C_{29}H_{36}O_{15}$	623.19760	623.19778	0.30	161.02360, 461.16642, 315.10787	Cisacteoside/Isoacteoside
13	35.890	$C_{29}H_{36}O_{14}$	607.20268	607.20302	0.56	161.02317, 133.02793, 445.16880	Kankanoside G
14	36.625	$C_{29}H_{36}O_{14}$	607.20268	607.20301	0.54	145.02852, 163.03922, 461.16573	Cis-Kankanoside G
15	38.403	$C_{31}H_{38}O_{16}$	665.20816	665.20826	0.15	161.02331, 461.16284, 133.02911	Cistubuloside B
16	39.275	$C_{29}H_{36}O_{14}$	607.20268	607.20306	0.62	161.02372, 133.02784, 445.17216	Isosyringalide-3′-α-L-rhamnose
17	40.175	$C_{29}H_{36}O_{14}$	607.20268	607.20300	0.53	145.02929, 461.16582, 163.04225	Syringalide A-3′-α-L–rhamnose
18	41.931	$C_{31}H_{38}O_{16}$	665.20816	665.20825	0.14	161.02361, 461.16594, 133.02833	Tubuloside B

4. Conclusion

The phenylethanoid glycosides in cistanche tubulosa were identified using high-resolution, high-accuracy Shimadzu Liquid Chromatography-quadrupole time-of flight mass

spectrometry (LCMS-9030). A total of 18 chemical components were identified using the Formula Predictor and ACD/Labs software, in combination with the UV data, retention time, and references, based on the primary and secondary high-resolution mass spectrometry information. The results showed that LCMS-9030 had a sub-ppm mass number accuracy and was a powerful tool for predicting molecular formula and deriving the structures of unknown compositions.