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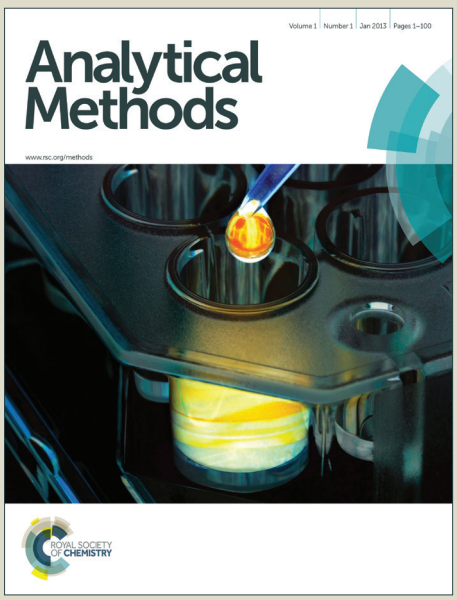

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Rapid discovery and global characterization of multiple constituents from Kai-Xin-San with an integrated MS^E data acquisition mode strategy based on ultra-performance liquid chromatography coupled to electrospray ionization/quadrupole-time-of-flight mass spectrometry

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Abstract

Kai-Xin-San (KXS) is a traditional Chinese medicine (TCM) formulae consisting of four herbs, Ginseng Radix, Polygalae Radix, Poria and Acori Tatarinowii Rhizoma, has been used to treat Alzheimer's disease and Parkinson's disease. In this study, UPLC/QTOF MS system with automated MS^E (E represents collision energy) data analysis software (MetaboLynx^{TM}) was used to analyze and identify the chemical components of KXS. Mass spectrometry was performed in a full-spectrum (m/z 100-2,000) mode using an MS^E acquisition of both molecular and fragment ion data at low (10-30 eV) and ramped (30-50 eV) collision energies. With the use of this approach, a total of 107 compounds were identified from KXS; 32 compounds were from Ginseng Radix; 19 compounds were from Poria; 33 compounds were from Polygalae Radix; 21 compounds were from Acori Tatarinowii Rhizoma, respectively. The developed method is fast, accurate and reliable due to its high resolution and high efficiency characteristics as a result of an integrated MS^E data acquisition mode strategy based on UPLC/QTOF MS that is powerful tool for global detection and identification of complex components in herbal prescriptions. The results indicated it provided helpful chemical information for further pharmacology and active mechanism research on KXS and other TCMs.

Keywords

Ultra-performance liquid chromatography coupled to electrospray ionization/quadrupole-time-of-flight mass spectrometry; Kai-Xin-San; constituents; identification; traditional Chinese medicine

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1. Introduction

Traditional Chinese medicine (TCM) preparations have been widely used and become increasingly popular worldwide. The comprehensive identification of the chemical components in TCMs is important for revealing their therapeutic effects [1]. However, the detection and structural characterization of each chemical constituent contained in herbal prescriptions are often unrealistic and unpractical. The development of sensitive, reliable analytical methods and technology have been proven to be efficient tools for the rapid on-line analysis of the known compounds and elucidation of unknown compounds in complex TCM. Recently, high-resolution ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/QTOF MS) has improved the analytical ability for the study of complex herbal medicine [2-5]. The well-developed UPLC/QTOF MS system renders it possible to launch a more comprehensive and thorough chemical profiling by utilizing versatile ionization techniques and/or different ion modes. In particular, it provides accurate precursor and/or product ions information with a mass error of less than the permitted ppm, which substantially enhances the characterization reliability of constituents [6]. Substantial progress has been made in using the LC/MS-based fingerprint technique in conjunction with automated MS^E (E represents collision energy) data analysis software (MetaboLynx^{TM}). MS^E technique can be used to predict the structures of unknown compounds in TCM based on the accurate molecular weight and the relationship between precursor and product ions [7-9].

Kai-Xin-San (KXS), firstly recorded in the Chinese ancient medical prescription book *Qian-Jin-Yao-Fang* around 1300 years ago, is a TCM formula consisting of four herbs, Ginseng Radix, Polygalae Radix, Poria, and Acori Tatarinowii Rhizoma (3:3:2:2) [10]. It has been used for thousands of years in China and other Asian countries to treat Alzheimer's disease, Parkinson's disease, etc [11-13]. Pharmacological studies showed that KXS had anti-oxidative, anti-depressive, and learning and memory improvement activities [14,15]. Recently, chemical analysis of KXS has rarely been reported. Lv determined the polygalaxanthone III, ginsenoside Rb1, ginsenoside Rd, ginsenoside Re, and ginsenoside Rg1 in the plasma of rat and beagle dog after oral administration of KXS by ultra-fast liquid chromatography with tandem mass spectrometry [16]. However, the studies of the chemical components in KXS have been mainly based on identification of chemical constituents in individual herb, further investigation was hindered due to the limited knowledge of its chemical components. An integrated MS^E data acquisition mode strategy based on UPLC/QTOF MS is powerful and reliable for global detection and identification of complex components in herbal prescriptions. Therefore, in this study, UPLC/QTOF MS method with automated MS^E data analysis was firstly developed to systematically characterize the chemical constituents of KXS.

2. EXPERIMENTAL

2.1 Materials

Acetonitrile and methanol (HPLC-MS grade) were purchased from Merck (Darmstadt, Germany). Deionized water

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(18.2 MΩ) was further purified using a Milli-Q system (Millipore, Billerica, USA). Leucine-enkephalin was obtained from Sigma-Aldrich. Poria, Ginseng Radix, Polygalae Radix, and Acori Tatarinowii Rhizoma were purchased from Harbin Tongrentang Drug Store (Harbin, China), and authenticated by Prof. Xijun Wang, Department of Pharmacognosy of Heilongjiang University of Chinese Medicine. Voucher specimens were deposited at the authors' laboratory.

2.2 Preparation of KXS samples

The KXS was prepared in the following procedure: Ginseng Radix, Poria, Polygalae Radix, Acori Tatarinowii Rhizoma in proportion 3:3:2:2, were ground into crude powders, mixed, and then reflux extraction in a rotary evaporator with 6 times volume of 70% ethanol for 2 h twice, temperature was set at 24 °C, then the filtrate was dried under freeze-drying. An aliquot of 0.1 g of each powder was dispersed in 10 mL 20% aqueous methanol (v/v) and ultrasonicated in a water bath for 10 min at room temperature to prepare solutions after filtration of the supernatant through a 0.22-μm filter membrane and 5 μL of the filtrate was injected into the UPLC/QTOF MS system for analysis.

2.3 Instrumentation and chromatography conditions

Chromatographic separation was performed using an UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent delivery system and a photodiode-array detector. ACQUITY ^{TM} UPLC HSS T_3 Column (2.1×100 mm, 1.8 μm, Waters Corporation, Milford, USA) was used for the analysis. The column temperature was set at 40 °C. The flow rate was 0.3mL/min. The optimal mobile phase consisted of A (HCOOH: H_2O=0.1: 100, v/v) and B (HCOOH: CH_3CN= 0.1:100, v/v). The elution conditions were optimized as follows: 0-1.0 min, 2-20 % A; 1.0-4.0 min, 20-21% A; 4.0-6.5 min, 21-30% A; 6.5-9.5 min, 30-35% A; 9.5-14.0 min, 35-50% A; 14.0-16.0 min, 50-52 % A; 16.0-16.5 min, 52-82 % A; 16.5-19.0 min; 82-83 % A; 19.0-19.5 min, 83-100 % A; 19.5-20.0 min, 100-2 % A; 20.0-21.0 min, 2 % A.

2.4 Mass spectrometry

The MS instrument used was a Waters Synapt ^{TM} High Definition TOF Mass system (Waters Corp., USA) equipped with an ESI source in both positive and negative ion modes. The MS source temperature was set at 110 °C, and the desolvation temperature was set at 300 °C with desolvation gas flow at 600 L/h. The capillary voltage was 3 kV. The mass spectra were recorded across the range of m/z 100 to 2700 Da, with accurate mass measurement of all mass peaks. The collision energy was set as 10-30 eV for low-energy scans, and 30–50 eV for high-energy scans. The instrument was controlled by Masslynx 4.1 (Waters Corp.).

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2.5 Data processing

LC-MS data of all determined samples were further processed by Markerlynx V4.1 software (Waters Corporation, Milford, USA) for peak detection and peak alignment. The method parameters for data processing were set as follows: retention time range 0.1-16.0 min, mass range 100-2000 Da, retention time tolerance 0.2 min, mass tolerance 0.05 Da, noise elimination level 6; peak intensity threshold 50. The peak width at 5% height and the peak-to-peak baseline noise were automatically determined. Pareto scaling of data was performed prior to analysis which generated the less noise level than other scaling method.

3. Results and discussion

To improve detection, the following MS conditions were optimized: formic acid concentration in mobile phase, ion mode, desolvation temperature, capillary voltage, and desolvation gas flow, etc. The reference compounds were used as test compounds to optimize the MS conditions. A positive and negative ESI source was chosen based on preliminary experiments. These optimal MS conditions were used in all further experiments. We developed a broad-spectrum screening method on an UPLC/QTOF MS with collision energy function (MS^E mode and accurate mass) which provides a unique way to incorporate fragment ion information without the need of precursor ion selection. Identification was based on the presence of one characteristic m/z ion obtained with the low collision energy function and at least one fragment ion obtained with the high collision energy function, both with mass errors of less than 5 ppm permitted. The molecular formulas with relative mass error between the measured and theoretical mass within 5 ppm were chosen and then applied to MassFragment analysis for further confirmation of their structures. High- and low-energy MS^E tool generates product ion spectra which were successfully applied to structural elucidation of detected KXS.

Detailed clarification of the chemical constituents in KXS is essential for holistic quality control. In order to complete the comprehensive characterization, multiple approaches, including database searching, reference standard comparison, and QTOF-MS and MS/MS data analysis, were employed for structural characterization of the KXS constituents. The chemical formula of an unknown structure was deduced based on the high-accuracy $[M-H]^-/[M+HCOO]^-$ (in the negative ion mode) or $[M+H]^+/[M+Na]^+$ (in positive ion mode) precursor ion. The compound(s) matching the determined chemical formula and considered to undergo the observed MS/MS fragmentation was selected as the final identity. As a result, a total of 107 compounds, were identified or tentatively characterized from the constituents of KXS. Information regarding the 107 constituents, such as the tR (min), identity, observed m/z values, mass error (in ppm), molecular formula, and botanical source, is offered in supplementary table 1. The ESI base peak ion (BPI) chromatogram of the KXS by UPLC/QTOF MS is shown in Figure 1. By UPLC/QTOF MS analysis, a total of 107 compounds including 61 in the negative ion mode and 59 in the positive ion mode were identified from KXS, 32 compounds including 15 ginsenosides (44, 49, 51, 54-61, 63, 64, 77, and 80)

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were from Ginseng Radix; 19 compounds including 7 triterpenoids (78, 79, 89, 91-92, 98, and 106) were from Poria; 33 compounds were from Polygalae Radix; 21 compounds were from Acori Tatarinowii Rhizoma. Generally, MS^E provides sufficient full scan MS and MS/MS spectral information. The characteristic MSn fragments of the 107 compounds are shown in supplementary table 2.

All information of MS data obtained from the robust UPLC-ESI-Q-TOF-MS analysis which was performed using the aforementioned protocol indicated the retention time and precise molecular mass and provided the MS/MS data which was necessary for the structural identification. Taking an example, in ESI negative ion mode, at low CE voltage, peak 56 produced aprominent $[M-H]^-$ ion atm/z 1107.5961. At high CE voltage, it produced fragment ions at m/z 945$[M-H-(Glc-H_2O)]^-$, 783$[M-H-2(Glc-H_2O)]^-$ and 459$[M-H-4(Glc-H_2O)]^-$ (Figure 2) , respectively. Thus, compound 56 was consistent with the MS/MS information of and tentatively identified as Ginsenoside Rb1 by comparing with a reference standard. Peak 49 gave an $[M-H]^-$ ion at m/z 799.4906, as shown in Table 1. In the high CE scan, 637$[M-H-(Glc-H_2O)]^-$, 475$[M-H-2(Glc-H_2O)]^-$,161$[M-H-Glc-C_{30}H_{50}O_3]^-$ were observed as the main fragmentation pathways (Figure 3), which was identical to that of Ginsenoside Rf. Peak 55 at 10.79 min gave an $[M+H]^+$ ion at m/z 605.4430. In the high CE scan, 587$[M+H-H_2O]^+$, 515$[M+H-C_5H_{12}-H_2O]^+$, 425$[M+H-Glc]^+$,407$[M+H-Glc-H_2O]^+$ were observed as the main mass fragments (Figure 4). The result suggested compound 55 might be a Ginsenoside Rh3. Peak 88 gave an $[M-H]^-$ ion at m/z 469.3318. It produced a similar MS/MS spectrum to that of 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid (Figure 5). Peak 23 at 2.41 min gave an $[M-H]^-$ ion at m/z 547.1663. In the high CE scan, it showed main fragment ions at m/z 385.1013, 367.1024, 325.0927 and 223.0574, 205.0497 (Figure 6). All the ions were similar to those of compound Sibiricose A1. By comparing the retention time with that of Sibiricose A1, compound 23 was tentatively identified as Sibiricose A1. Compound 73 were identified to be α-asarone, which was confirmed by comparison with a reference compound. In the high CE scan, it produced the same fragment ions at m/z 194.0910, 179.0678, 151.0734, and 121.0586 as those of α-asarone (Figure 7). Similarly, other compounds could also be characterized according to the above-mentioned methods. The results demonstrate the potential of UPLC/QTOF MS coupled with the MarkerLynx$^{(TM)}$ software and MS^E tool as an efficient and convenient method to screen and identify constituents in herbal medicine.

4. Conclusion

In this work, we demonstrated the use of UPLC/QTOF MS coupled with automated MS^E data analysis for the first time for structural characterization of global compounds in KXS. Using MS^E approach and UPLC/QTOF MS technique, a total of 107 compounds were characterized tentatively, 32 compounds including 15 ginsenosides were from Ginseng Radix; 19 compounds including 7 triterpenoids were from Poria; 33 compounds were from Polygalae Radix; 21 compounds were from Acori Tatarinowii Rhizoma, respectively. These findings provided informative groundwork for further pharmacological studies of KXS prescription. This strategy could greatly increase the

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knowledge of the components of TCMs, we expected that approach would be useful for the screening and characterization of compounds in other famous herb medicines.

Acknowledgments

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Competing financial interests

The authors declare no competing financial interests.

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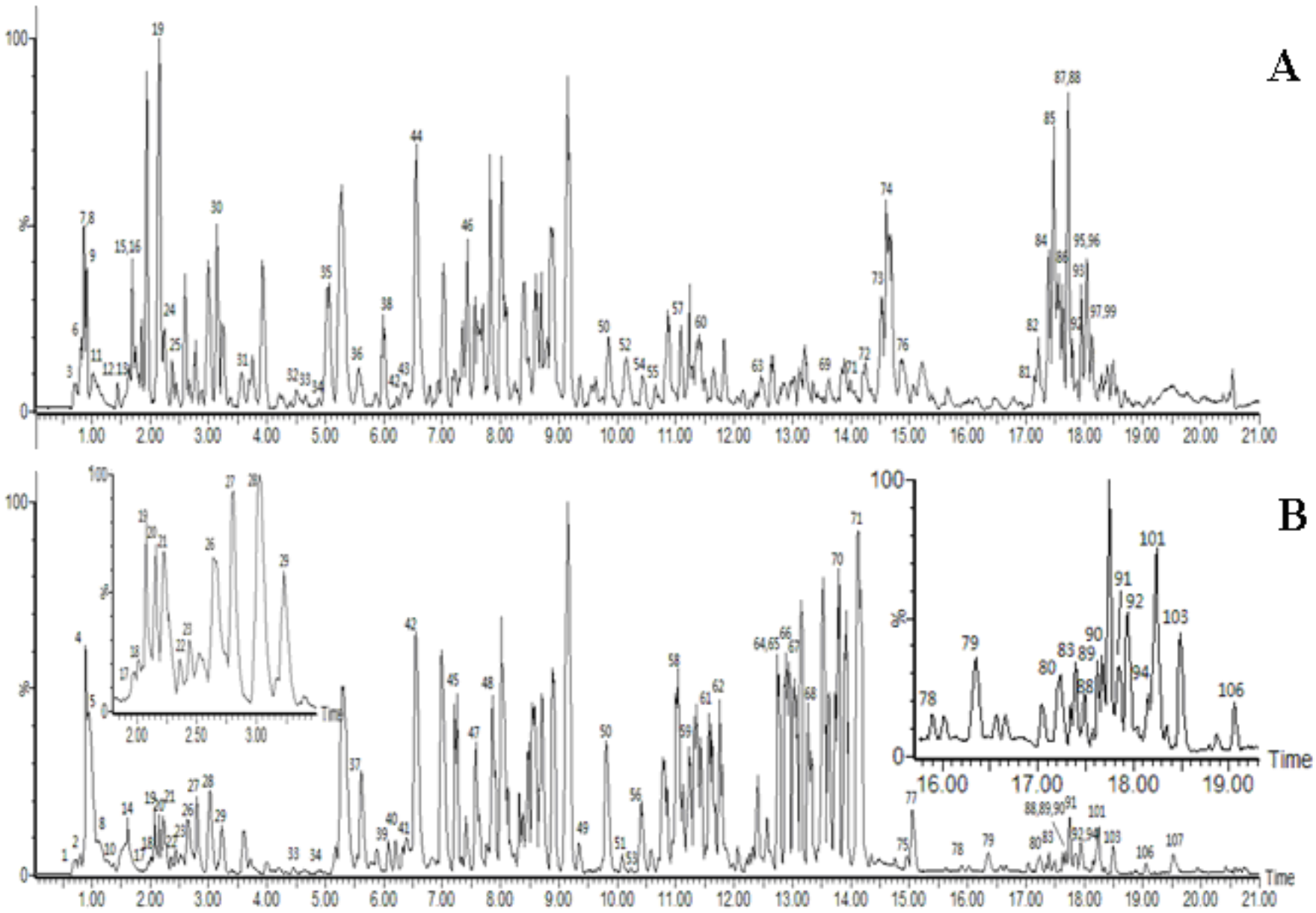

Figure 1. ESI base peak ion (BPI) chromatogram of the KXS analyzed by UPLC/QTOF MS.

Positive ion mode (A); negative ion mode (B). (The peak numbers were displayed in Table 1)

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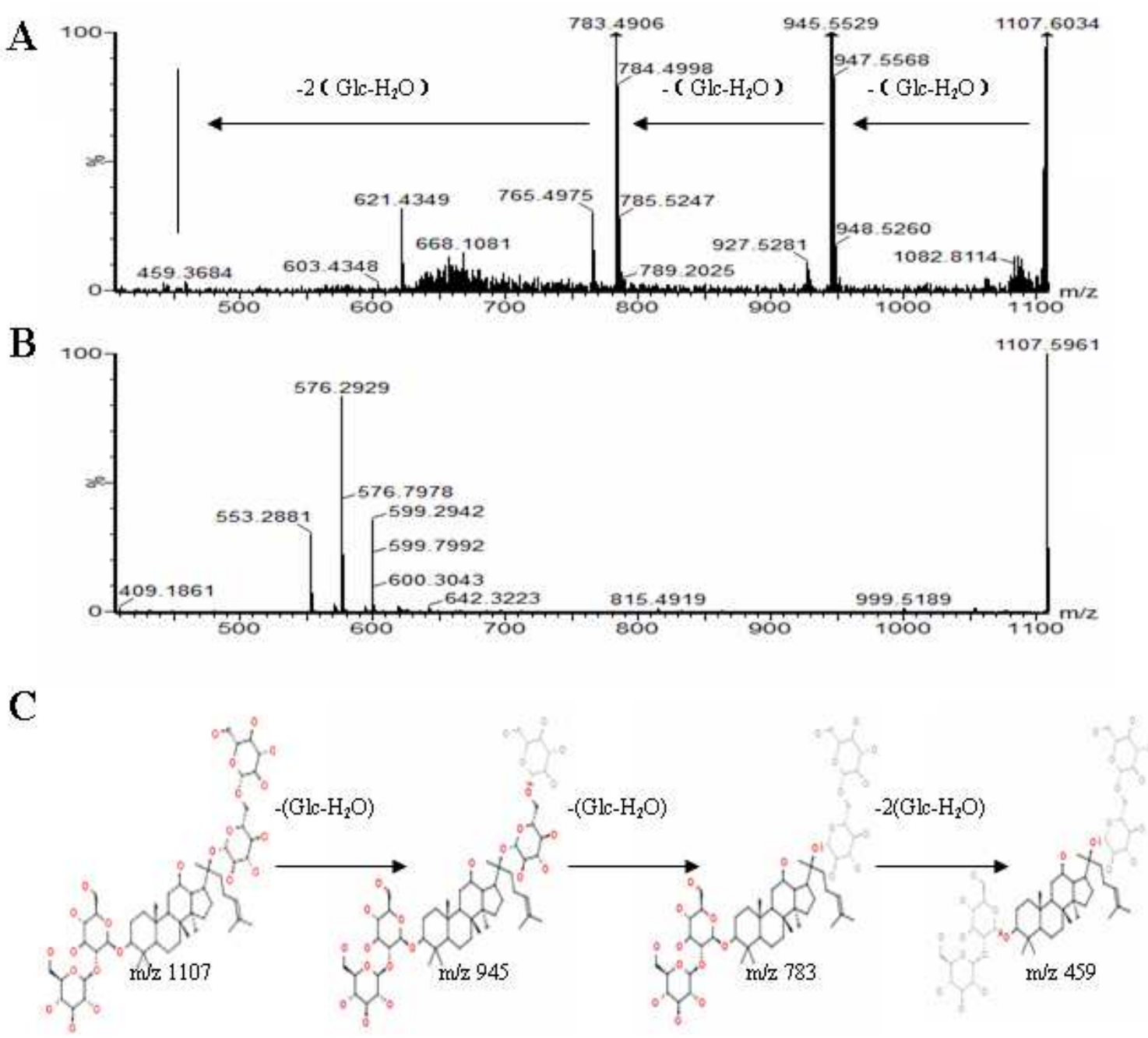

Figure 2. High-resolution MS and MS/MS spectra and fragmentation pathways of ginsenoside Rb1 in negative mode.

The mass spectrum at high collision energy (A); the mass spectrum at low collision (B); the proposed fragmentation pathways of ginsenoside Rb1 (C).

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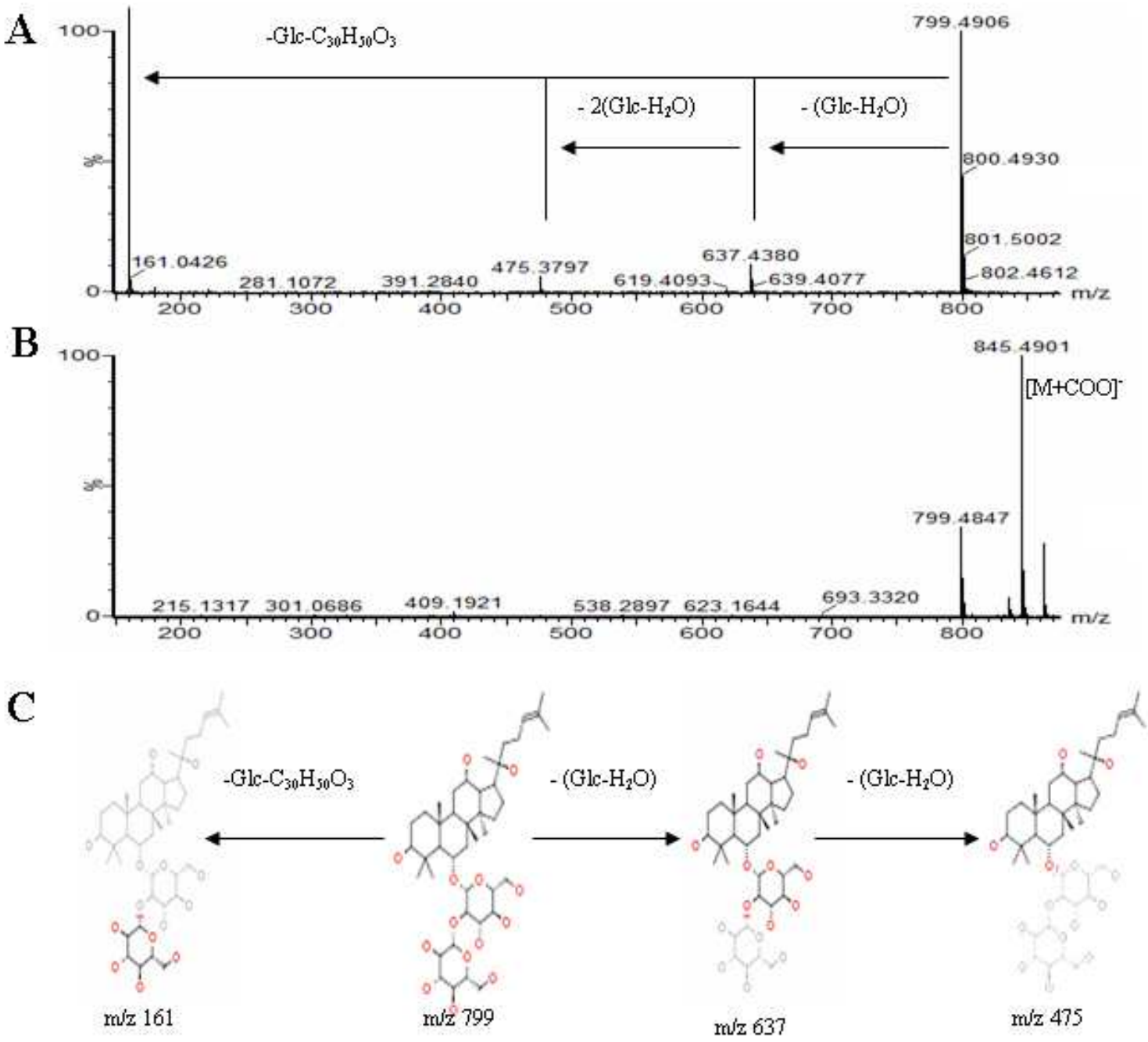

Figure 3. High-resolution MS and MS/MS spectra and fragmentation pathways of ginsenoside Rf in negative mode. The mass spectrum at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of Ginsenoside Rf (C).

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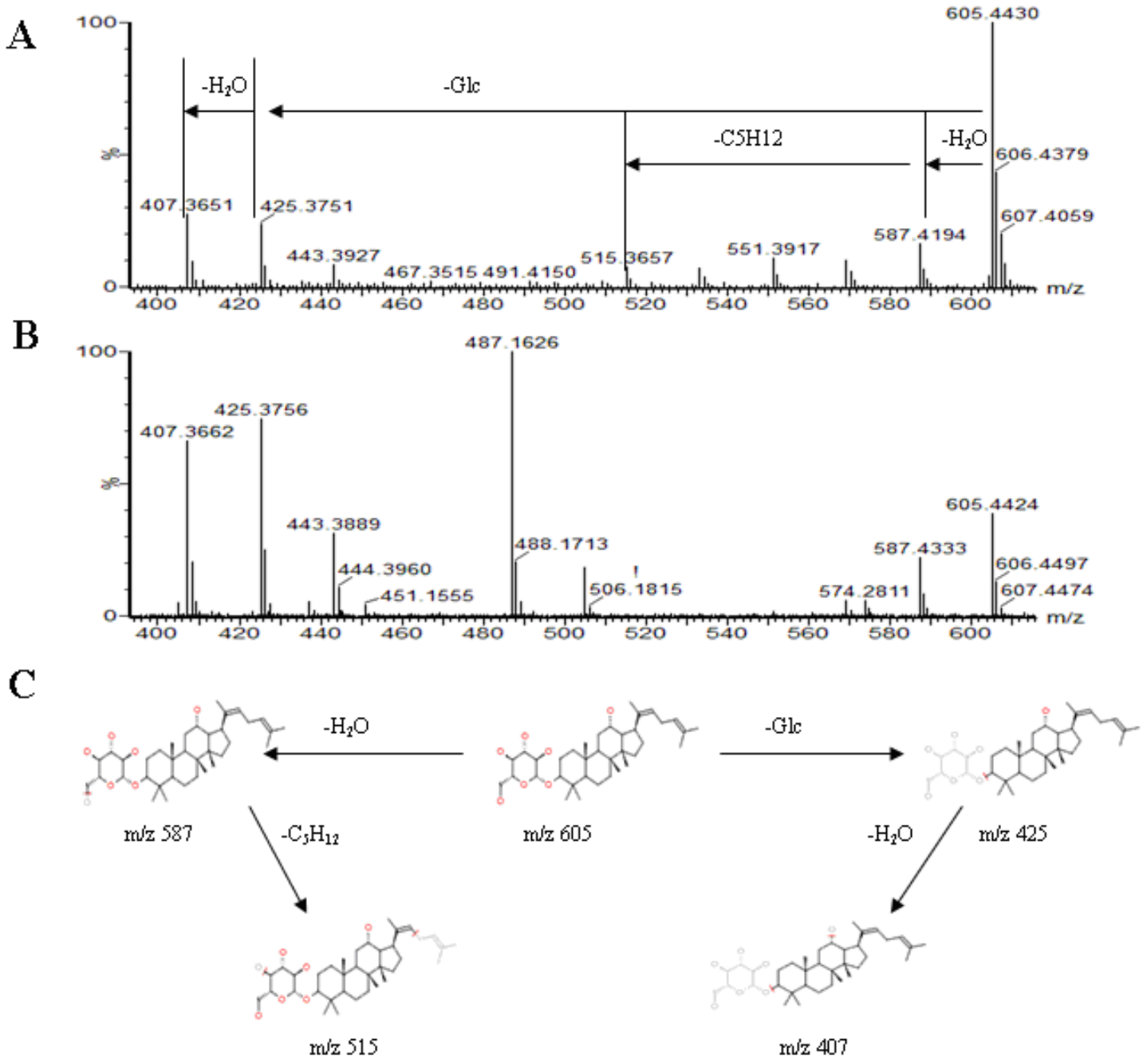

Figure 4. The MSE spectra and fragment assignment of ginsenoside Rh3 in positive mode.

The mass spectrum at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of ginsenoside Rh3 (C).

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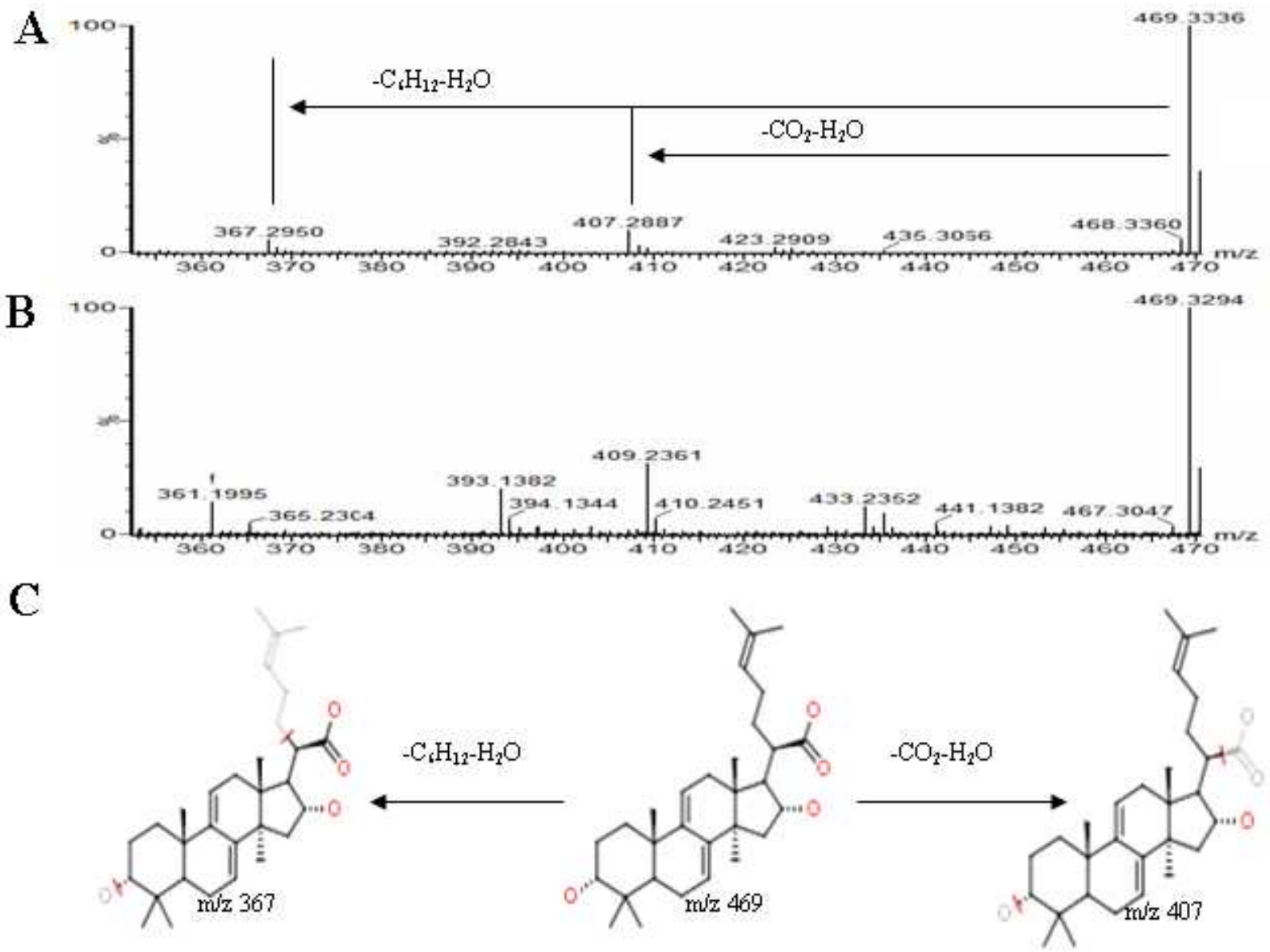

Figure 5. The mass spectra of 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid in negative mode. The mass spectrum at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of 3β,16α-Dihydroxylanosta-7,9(11),24-trien-21-oic acid (C).

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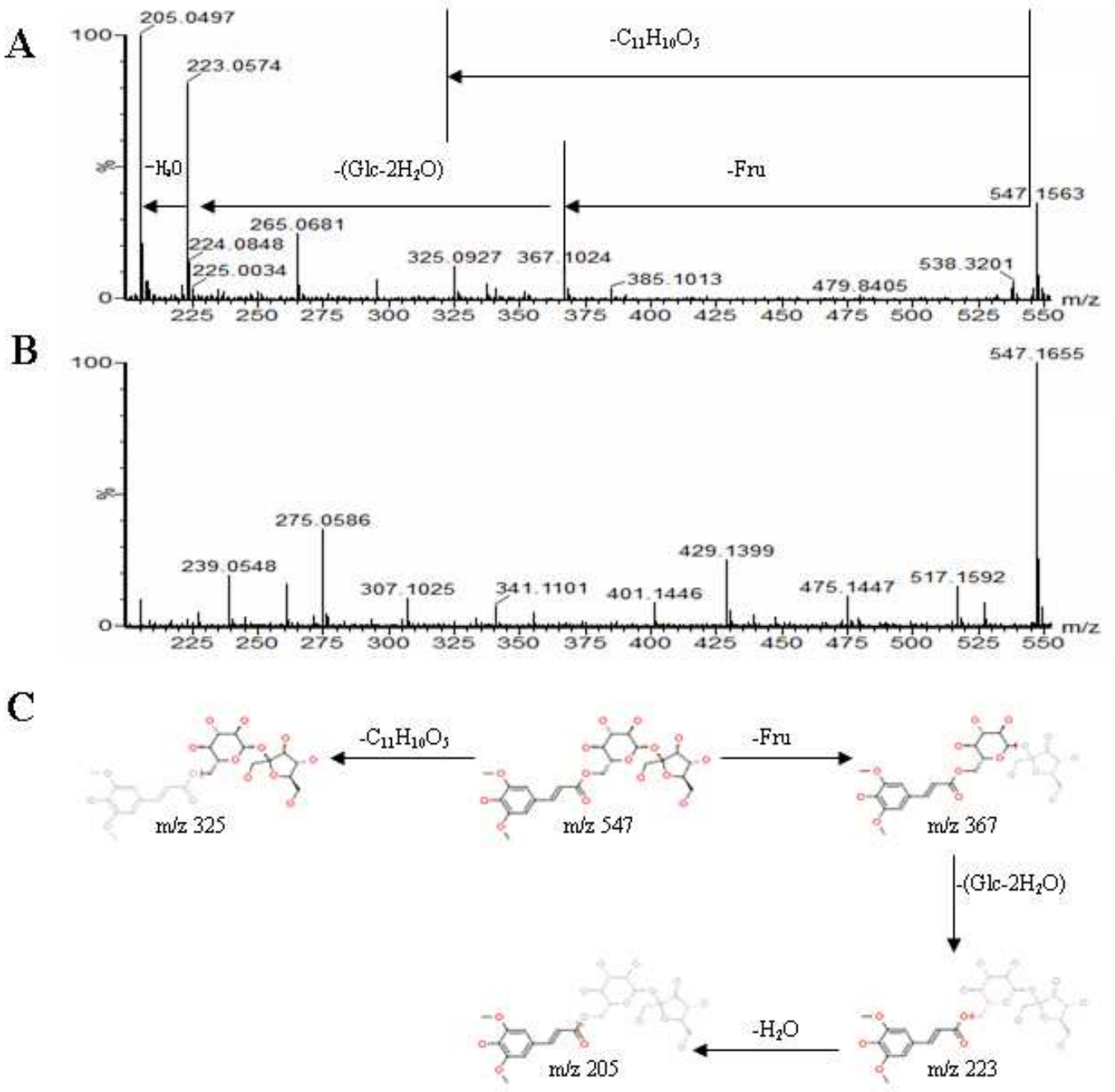

Figure 6. High-resolution MS and MS/MS spectra and fragmentation pathways of sibiricose A1 in negative mode. The mass spectrum at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of sibiricose A1 (C).

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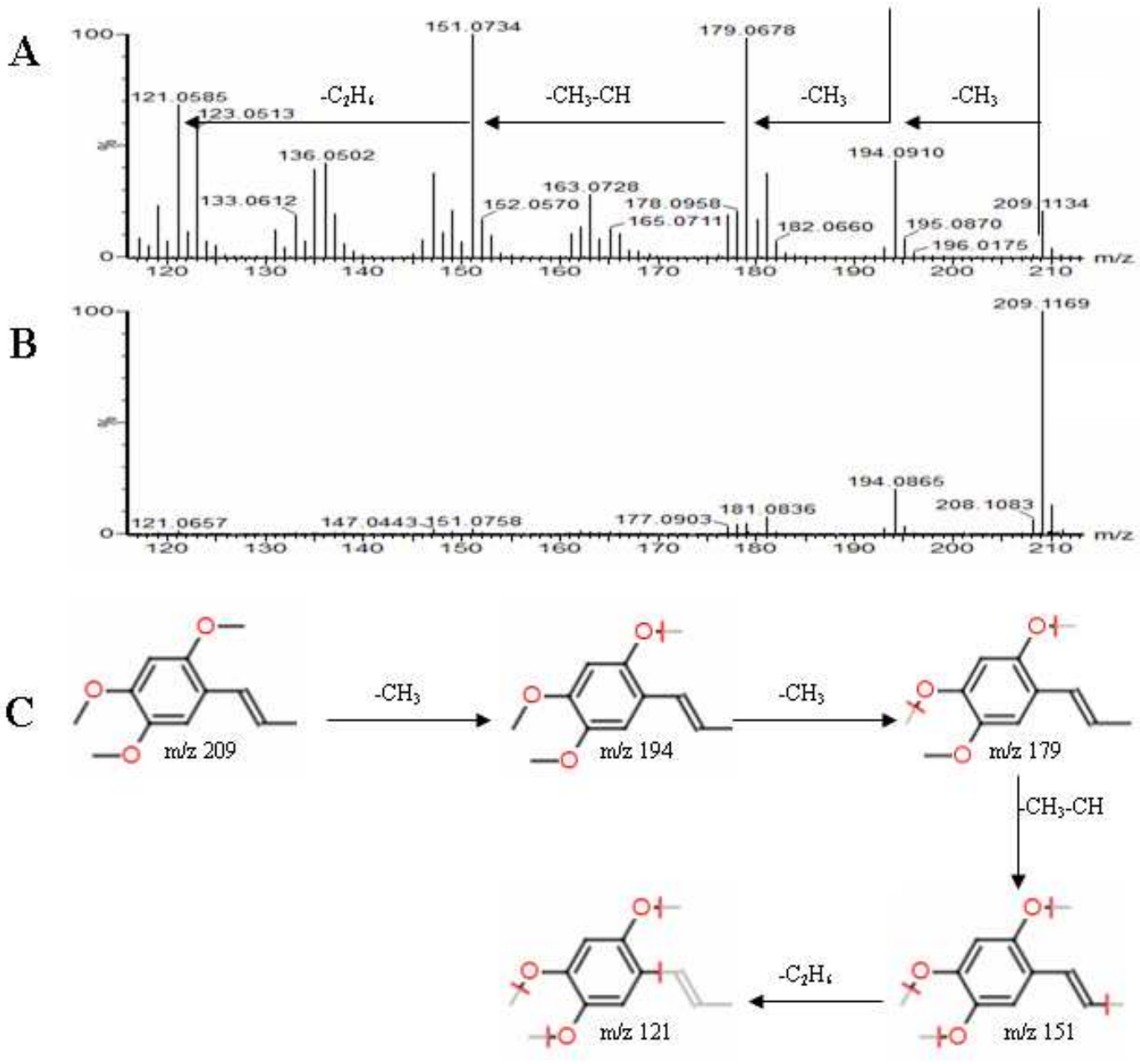

Figure 7. The MSE spectra and fragment assignment of α-asarone in positive mode.

MS^E spectra and fragment assignment at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of α-asarone (C).

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