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Short communication

Rapid separation and characterization of active flavonolignans of *Silybum marianum* by ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry

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ABSTRACT

Ultra-performance liquid chromatography (UPLC) interfaced with the electrospray ionization (ESI) tandem mass spectrometer (MS^n) was developed for the simultaneous determination of silychristins A (**1**) and B (**2**), silydianin (**3**), silybins A (**4**) and B (**5**), and isosilybins A (**6**) and B (**7**), major bioactive flavonolignans in silymarin, a herbal remedy derived from the milk thistle *Silybum marianum*. In this study, the seven major active flavonolignans including the diastereomers **1**/**2**, **4**/**5**, and **6**/**7** were completely separated using UPLC with an ACQUITY UPLC C_{18} column and a MeOH/water/formic acid mobile phase system. The collision-induced dissociation (CID) MS^n spectra of these flavonolignans were studied systematically using ESI-MS. The results with the present methodology show that UPLC–MS^n can be useful for general screening of active natural products from plant extracts and for the specific quality control of silymarin.
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1. Introduction

Silymarin, the extract of *Silybum marianum* (L.) Gaertn. (Asteraceae), has been used for centuries as a natural remedy in the supportive therapy of liver diseases and this traditional medicinal use has been supported by clinical trials [1–4]. Recent reports have demonstrated that silybin, the major hepatoprotective flavonolignan component of silymarin, also has exceptionally high anti-tumor activity and many other pharmacological activities such as anti-inflammatory and anti-fibrotic effects [5,6]. Natural silymarin is a mixture of flavonolignans, which include diastereomers silychristins A (**1**) and B (**2**), silybins A (**4**) and B (**5**), isosilybins A (**6**) and B (**7**), and silydianin (**3**) (Fig. 1), as major bioactive components [7–9].

Most methods used to analyze silymarin involving reversed phase high performance liquid chromatography (RP-HPLC) or HPLC-collision-induced dissociation (CID)-MS/MS have been reported [10–12]. However, the separation of silychristin B and silydianin by HPLC was not achieved in these reports. So, it is very important for the establishment of a rapid and selective analytical method to characterize and quantify each component of silymarin, and furthermore, to monitor these active components in plasma or other pharmacology study.

Traditional chromatographic separation of multi-component analytes on HPLC is relatively time-consuming. The introduction of ultra-performance liquid chromatography (UPLC) has made it possible to shorten the analysis time while maintaining the resolution and increasing peak capacity and sensitivity [13,14]. Since the advent of UPLC systems, much interest has been generated regarding the prospect of achieving increased resolution, throughput and sensitivity for small-particles column [15–22]. Many applications utilizing UPLC systems in proteins analysis, pharmaceutical analysis has shown the advantages described above. However, little research was performed on the use of UPLC in silymarin analysis. Thus, the aim of this paper was to develop a rapid, sensitive and accurate method for the determination of silymarin, using ultra-performance liquid chromatography–tandem mass spectrometry for separation and detection.

In this study, we report the complete separation of these seven active flavonolignans by UPLC with photodiode array detection (DAD). Moreover, the product ions from CID-MS^n spectra of these flavonolignans were systematically analyzed through electrospray ionization mass spectrometry. Our primary objective was to attain a more detailed analysis of fragmentation in CID using mass spectrometric technique and to distinguish between the silychristins (**1**, **2**), silydianin (**3**), silybins (**4**, **5**), and isosilybins (**6**, **7**) for online identification.

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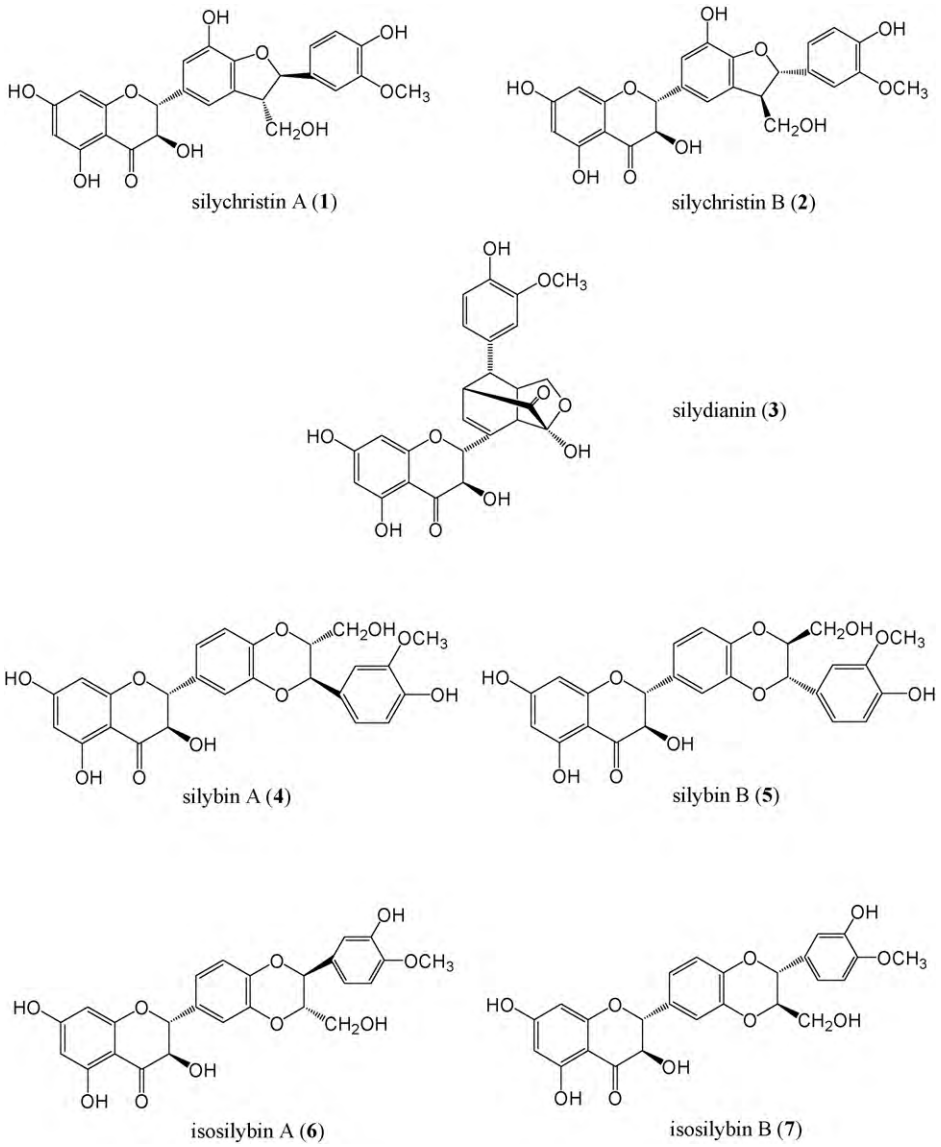

Fig. 1. Chemical structure of compounds **1**–**7**.

2. Experimental

2.1. General experimental procedures

The instruments used in this study were Waters UPLC (for analysis HPLC) instrument (Waters Corporation, Milford, MA, USA), LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific, Madison, WI, USA).

2.2. Reagents and materials

HPLC-grade MeOH (Merck, for LC–MS) were purchased from Merck Co. (Darmstadt, Germany). Reagent-grade formic acid (98%) was purchased from Fluka Chemical Corporration (Ronkonkoma, NY, USA). Ultra-pure water was prepared using a Millipore Milli-Qpurification system (Millipore Corporation, Billerica, MA, USA). Milk thistle (*S. marianum*) extract powder was supplied by Kangning Co., Ltd. (Jiangsu Province, China).

2.3. Sample preparation for UPLC–MS

Extract powder (1 mg) was dissolved in MeOH (10 mL). The solution was filtered using a 0.22 μm filter and then diluted with HPLC-MeOH to 100 ppm.

2.4. UPLC analysis

UPLC was performed using a Waters UPLC system. Chromatographic separation was performed on an ACQUITY UPLC C_{18}, (1.7 μm, 50 mm × 2.1 mm i.d.) (Waters Corporation, Milford, MA, USA). Mobile phase A was water containing 0.1% formic acid. Mobile phase B was MeOH containing 0.1% formic acid. The column temperature was ambient. The UPLC flow rate was 0.25 mL/min. A sample solution of 5 μL was injected into the UPLC system. A mobile phase gradient was used with the percentage of B in A varying as follows: initial concentration, 30% B; 7 min, 50% B; 10 min, 50% B, 13 min, 30% B.

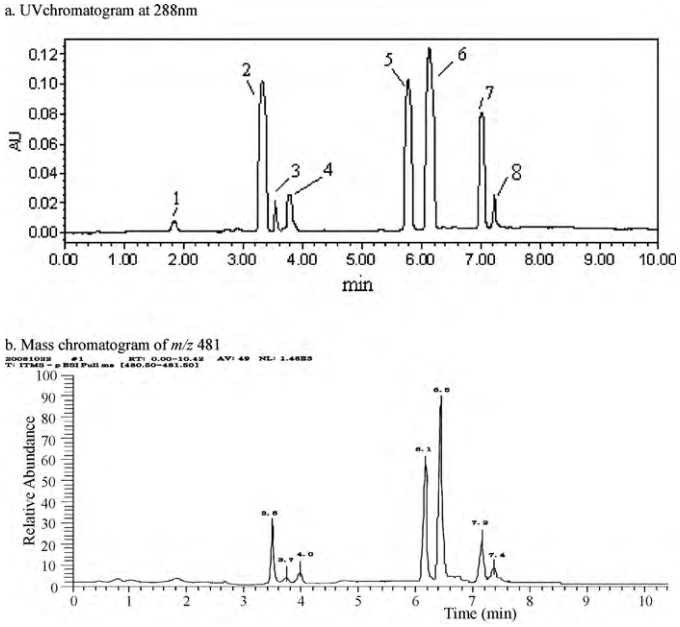

Fig. 2. (a and b) UPLC–UV–MS chromatogram of silymarin extract.

2.5. ESI-MS analysis

CID-MS^n experiments were performed on an electrospray ionization mass spectrometer (LCQ Fleet Ion Trap MS^n, Thermo Scientific, Madison, WI, USA). The negative ESI conditions were as follows. The spray voltage was set at −5 kV and the spray was stabilized with a nitrogen sheath gas (30 arb/min). Capillary temperature was 300 °C. The isolation width of precursor ions was 2.0 mass units. Ions were obtained in the range of *m*/*z* 100–1000. The scan time was equal to 100 ms and the collision energy (CE) was optimized according to compounds. Data were treated with the Xcalibur software.

3. Results and discussion

Fig. 2 shows the chromatograms obtained from silymarin analyzed using the method presented here. The wavelength for detecting these flavonolignans was set at 288 nm, and UV spectra from 210 to 500 nm were also recorded for peak characterization with DAD. In addition, in the mass chromatogram, ($[M-H]^-$) at *m*/*z* 481 was selected to monitor for all seven flavonolignans since the seven compounds have the same molecular weight (molecular formula $C_{25}H_{22}O_{10}$). The peaks numbered from 2–8 were completely separated. These peaks were identified as peak 2: silychristin A (**1**), peak 3: silydianin (**3**), peak 4: silychristin B (**2**), peak 5: silybin A (**4**), peak 6: silybin B (**5**), peak 7: isosilybin A (**6**) and peak 8: isosilybin B (**7**), respectively, by using the reference standards and comparing their HPLC retentions times as well as relative intensities of their fragment ions.

In the HPLC-MS analysis of silymarin reported previously [10,11], formic acid was used as a mobile phase modifier. However, silychristin B (**2**) and silydianin (**3**) are overlapped. Therefore, to obtain a good resolution of adjacent peaks in a reasonably short analysis time, different mobile phases and column compositions (such as methanol and water (3:7), MeCN/MeOH/H_2O (2.5:45:52.5), methanol (A) and aqueous dioxane (B) (90% water + 10% dioxane), methanol–water containing 0.1% formic acid (45:55, v/v) of the mobile system and Agilent XDB-C18, Nucleosil 100-3C_{18} HD, Shim-pack VP-ODS, Phenomenex C_{18} of column) were screened. We found that the most suitable eluting solvent system was MeOH and 0.1% aqueous formic acid, and the best column was ACQUITY UPLC C_{18} HD (1.7 μm, 50 mm × 2.1 mm i.d). The UPLC gradient profile is described in experimental section. The optimized UPLC conditions permitted a good separation of seven target flavonolignans in silymarin within 8 min.

The negative-ion mode of electrospray ionization (ESI) was selected for MS analysis, as it easily provided extensive information via CID fragmentations. The product ions spectra of the pseudomolecular ions $[M-H]^-$ were obtained by conducting CID-MS/MS experiments. In CID-MS^n experiments, product ions at *m*/*z* 463, 453, and/or 355, 301, which are key ions to discriminate between the seven target flavonolignans, were chosen as precursor ions, respectively.

Silychristins A and B (**1** and **2**) were detected at retention times (t_R) 3.31 and 3.82 min as peaks 2 and 4, respectively. In a CID-MS/MS experiment at *m*/*z* 481 ($[M-H]^-$), major product ions at *m*/*z* 463 $[M-H_2O-H]^-$ and 355 $[M-C_6H_6O_3-H]^-$ along with fragment ions at *m*/*z* 451 $[M-HCHO-H]^-$, 433 $[M-H_2O-HCHO-H]^-$, 419 $[M-CO_2-H_2O-H]^-$, 337 $[M-C_6H_6O_3-H_2O-H]^-$ and 325 $[M-C_6H_6O_3-HCHO-H]^-$ (Fig. 3) were observed. According to the pathway elucidated in Fig. 3, product ion at *m*/*z* 463 corresponded to the losses of H_2O (18 Da) at C-3 and C-4 or at C-8′ and C-9′. CID-MS^3 at *m*/*z* 463 showed three diagnostic fragment ions: *m*/*z* 445 ($[M_{463}-H_2O]^-$, base peak), 435 ($[M_{463}-CO]^-$), 419 ($[M_{463}-CO_2]^-$) and 337, and product ions at *m*/*z* 325 ($[M_{355}-CO]^-$) and 337 ($[M_{355}-H_2O]^-$, base peak) resulting from *m*/*z* 355. CID-MS^4 at *m*/*z* 445 showed four typical fragment ions: *m*/*z* 430 ($[M_{445}-{}^{\bullet}CH_3]^-$), 427 ($[M_{445}-H_2O]^-$), 417 ($[M_{445}-CO]^-$) and 401 ($[M_{445}-CO_2]^-$), while *m*/*z* 419 showed five diagnostic product ions: *m*/*z* 401($[M_{419}-H_2O]^-$), 389 ($[M_{419}-HCHO]^-$, base peak), 377, 335 and 283. We have proposed the structures of these product ions as shown in Fig. 3. However, it was not possible to define a difference between fragment patterns of the two diastereomers (**1** and **2**).

Peak 3 at t_R 3.54 min was detected as silydianin (**3**). The product-ion spectrum of the $[M-H]^-$ (at *m*/*z* 481) showed typical product ions at *m*/*z* 463 $[M-H_2O-H]^-$, 453 $[M-CO-H]^-$ (base peak), 437 $[M-CO_2-H]^-$, 409 $[M-CO-CO_2-H]^-$, 391 $[M-H_2O-CO-CO_2-H]^-$, 301, 179, 169 and 151, which different from compound **1** and **2**. CID-MS^3 with *m*/*z* 453 showed fragment ions at *m*/*z* 435 ($[M_{453}-H_2O]^-$), 425 ($[M_{453}-CO]^-$), 409 ($[M_{453}-CO_2]^-$), 391 ($[M_{453}-CO_2-H_2O]^-$), 381 ($[M_{453}-CO_2-CO]^-$, base peak), 327, 313 and 273. While *m*/*z* 463 showed diagnostic fragment ions at *m*/*z* 445 ($[M_{463}-H_2O]^-$, base peak), 435 ($[M_{463}-CO]^-$), 419 ($[M_{463}-CO_2]^-$), 401 ($[M_{463}-CO_2-H_2O]^-$), 391 ($[M_{463}-CO_2-CO]^-$), 377, 353, and 335.

Silybins A and B (**4** and **5**) were detected at t_R 5.82 and 6.20 min as peaks 5 and 6, respectively. The deprotonated molecular ion $[M-H]^-$ yielded six predominant fragments at *m*/*z* 463 $[M-H_2O-H]^-$ (base peak), 453 $[M-CO-H]^-$, 355 $[M-C_6H_6O_3-H]^-$, 301, 283, and 257 in the CID-MS/MS experiment. The CID-MS^3 from *m*/*z* 463, 453, 355, and 301 showed many diagnostic fragment ions: *m*/*z* 445 ($[M_{463}-H_2O]^-$), 435 ($[M_{463}-CO]^-$), 433 ($[M_{463}-HCHO]^-$, base peak), 419 ($[M_{463}-CO_2]^-$), 353, 283 and 239 resulted from *m*/*z* 463, *m*/*z* 435 ($[M_{453}-H_2O]^-$, base peak), 409 ($[M_{453}-CO_2]^-$), 273, and 229 resulted from *m*/*z* 453, *m*/*z* 337 ($[M_{355}-H_2O]^-$), 327 ($[M_{355}-CO]^-$, base peak), 191, 177 and 175 resulted from *m*/*z* 355, *m*/*z* 283 ($[M_{301}-H_2O]^-$), 273 ($[M_{301}-CO]^-$, base peak) and 255 ($[M_{301}-H_2O-CO]^-$) resulted from *m*/*z* 301. CID-MS^4 at *m*/*z* 433 showed fragment ions at *m*/*z* 418 ($[M_{433}-{}^{\bullet}CH_3]^-$), 405 ($[M_{433}-CO]^-$) and 390 ($[M_{433}-{}^{\bullet}CH_3-CO]^-$).

Isosilybins A and B (**6** and **7**) were detected at t_R 7.01 and 7.22 min as peaks 7 and 8, respectively. The CID-MS/MS obtained from the precursor ion at *m*/*z* 481 showed major product ions at *m*/*z* 463 $[M-H_2O-H]^-$, 453 $[M-CO-H]^-$ (base peak), 437 $[M-CO_2-H]^-$, 301, 283 and 257, while the product ion at *m*/*z* 355 was not detected. In the CID-MS^3 experiment, *m*/*z* 445 ($[M_{463}-H_2O]^-$), 435

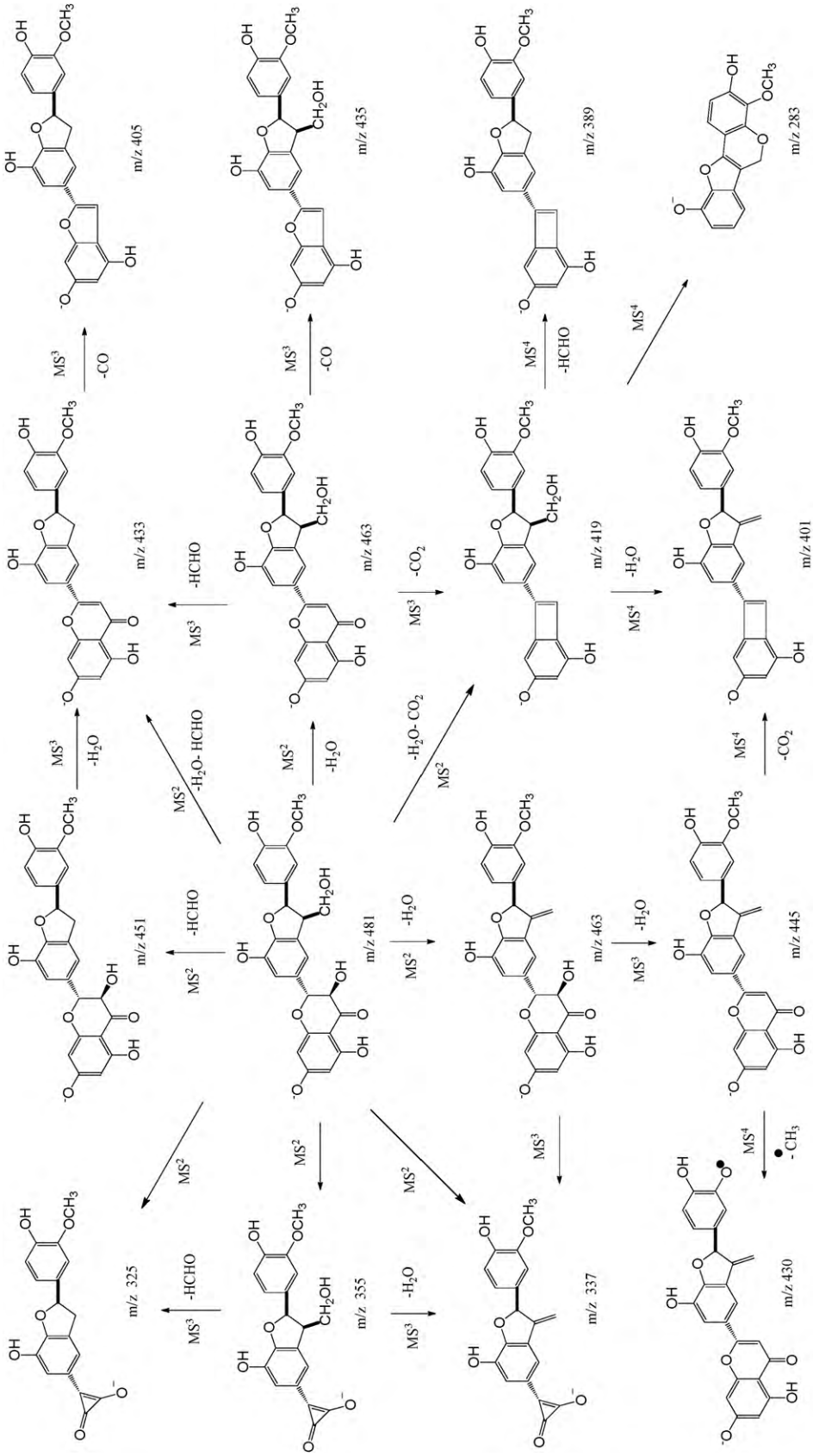

Fig. 3. Proposed fragmentation pathways of silychristins A (**1**) and B (**2**).

($[M_{463}-CO]^-$), 433 ($[M_{463}-HCHO]^-$), 419 ($[M_{463}-CO_2]^-$) and 283 resulted from m/z 463, with m/z 283 observed as the base peak, and m/z 435 ($[M_{453}-H_2O]^-$) was produced from m/z 453. The product ions at m/z 417 ($[M_{435}-H_2O]^-$), 285 (base peak) and 273 were produced from m/z 435 in the CID-MS^4 experiment.

The major distinction between isosilybins A and B (**6** and **7**) and silybins A and B (4 and 5) is the difference of peak intensity for the unique fragment ion at m/z 301, 453 and 463 and the absence of fragment ion of m/z 355 and 433 in the product ions spectrum of isosilybins A and B. The relative abundance of m/z 301 for silybins A and B is much higher than those observed in the product ion spectrum of isosilybins A and B. However, no observable difference was seen in the relative intensity of fragment ion of m/z 301 between silybins A and B or between isosilybins A and B. Fewer diagnostic fragment ions were produced from **6** and **7** than from **4** and **5**, and moreover, the characteristic MS^2 product ions at m/z 355 of **4** and **5** were not observed from **6** and **7**. The lose of methyl radical was observed in compound **1**, **2**, **4** and **5** but did not detected in compounds **3**, **6** and **7**. These are useful in identifying these seven active flavonolignans online.

4. Conclusions

In this paper, we established a simple and rapid analytical method that could simultaneously separate and determinate seven major bioactive flavonolignans in silymarin based on ultra-performance liquid chromatography/tandem mass spectrometry. It took less than 8 min to finish the sample analysis. Moreover, the detailed structures of fragment ions from CID-MS/MS, CID-MS^3 and CID-MS^4 of these flavonolignans were established by using the electrospray ionization mass spectrometry. Comparing to the conventional HPLC method, UPLC showed many advantages, including reduced run time, less solvent consumption and increased peak capacities. So, the UPLC-ESI-MS^n is a useful tool for identifying and elucidating the fragmentation pathway of natural products and will become very useful for quick screening active natural products from plant extracts or plasma samples in the pharmacokinetic study.

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