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Full Paper

Identification of Phenylethanoid Glycosides in Plant Extract of *Plantago asiatica* L. by Liquid Chromatography-Electrospray Ionization Mass Spectrometry

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The present work describes a liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) method for rapid identification of phenylethanoid glycosides in plant extract from *Plantago asiatica* L. By using a binary mobile phase system consisting of 0.2% acetic acid and acetonitrile under gradient conditions, a good separation was achieved on a reversed-phase C_{18} column. The $[M-H]^-$ ions, the molecular weights, and the fragment ions of phenylethanoid glycosides were obtained in the negative ion mode using LC-ESI-MS. The identification of the phenylethanoid glycosides (peaks 1—3) in the extract of *P. asiatica* L. was based on matching their retention time, the detection of molecular ions, and the fragment ions obtained by collision-induced dissociation (CID) experiments with those of the authentic standards and data reported in the literature.

Keywords *Plantago asiatica* L., phenylethanoid glycoside, liquid chromatography-electrospray ionization mass spectrometry

Introduction

The aerial parts of *Plantago asiatica* L. have been used as a diuretic, an anti-inflammatory and an anti-asthmatic drug in China and Japan.[1] The major bioactive constituents of *P. asiatica* L. are phenylethanoid glycosides, which are widely distributed in the plant kingdom,[2-10] and have been found to have various biological activities, such as anti-hepatotoxic,[11] anti-inflammatory, anti-nociceptive[12] and antioxidant[13,14] activities. Owing to these significant bioactivities, effective methods for the structural characterization and identification of phenylethanoid glycosides become necessary. However, such work requires relatively large sample sizes of purified compounds, and the use of conventional chromatographic methods for sample clean up and separation. These methods are often time consuming, and involve repeated chromatographic steps on silica gel and Sephadex LH-20 columns.[3-8] Furthermore, the hydroxyl groups in the molecules of phenylethanoid glycosides make these compounds strongly adsorbed onto the solid support during separation.[15] In recent years, high-speed counter-current chromatography (HSCCC) methods have become an effective alternative to the conventional chromatographic techniques for the separation of phenylethanoid glycosides.[15,16] However, structures of the purified compounds still need to be confirmed with other analytical tools such as mass spectrometry and nuclear magnetic resonance (NMR) for these methods. A simple, sensitive and less laborious analytical method for rapid identification of phenylethanoid glycosides in plant extracts is therefore necessary.

LC-MS methods have been proved to be a powerful analytical tool for rapid identification of non-volatile compounds in plant extract.[17-19] Wang *et al*.[19] successfully analyzed phenylethanoid glycosides in plant extract from *Cistanches deserticola* by using LC-ESI-MS. An LC-APCI-MS has also recently been reported by the authors of this paper.[16] However, no report has been published on the use of LC-ESI-MS for the identification of phenylethanoid glycosides in the aerial parts of *P. asiatica* L.

In this paper, we report a simple, direct and reliable LC-ESI-MS method for the rapid identification of phenylethanoid glycosides in the crude extract from *P. asiatica* L. The retention time, molecular weights and the characteristic fragment ions of three major phenylethanoid glycosides are presented and discussed

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in this paper. The structures of phenylethanoid glycosides identified in this investigation are showed in Figure 1.

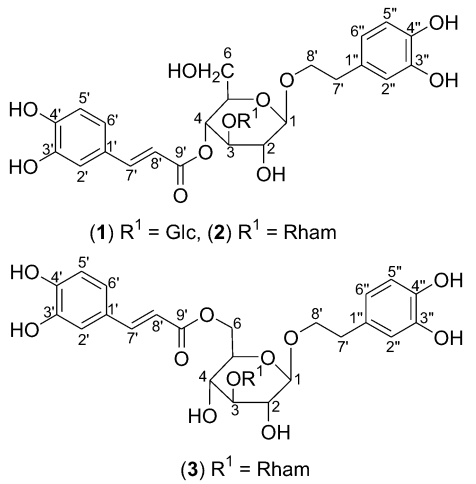


Figure 1 Structures of phenylethanoid glycosides in the aerial parts of *Plantago asiatica* L. identified in this study: plantamajoside (**1**), acteoside (**2**) and isoacteoside (**3**).

Experimental

Reagent and chemicals

Acetonitrile was of HPLC grade from Fisher Chemicals (USA), and all other reagents and solvents were of analytical grade from Beijing Chemicals (Beijing, China). Water was purified using a Milli-Q water purification system (Millipore, France). Acteoside and isoacteoside were separated and purified from the seeds of *Plantago psyllium* L. by the authors using HSCCC.[16] *P. asiatica* L. was purchased from Beijing TongRenTang Medicinal Store (Changchun, China).

Sample preparation

The aerial part of *P. asiatica* L. (5.2025 g) was soaked in two separate volumes of 100 mL of 80% ethanol for 12 h at room temperature. The two mixtures were combined and filtered through a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The filtrate was evaporated to 30 mL *in vacuo* at ＜40 ℃. The resulting aqueous solution was defatted twice, each with 30 mL of hexane. The remaining aqueous layer was extracted successively for three times, each with 20 mL of *n*-butanol. The *n*-butanol layers were combined and evaporated to dryness *in vacuo* at ＜40 ℃. The residue was then dissolved in 10 mL of methanol and filtered through a 0.45 μm membrane before being used for the LC-ESI-MS experiments.

LC-ESI-MS for identification

A high performance liquid chromatography (HPLC) system consisting of a Waters 2690 pump equipped with a gradient controller, an automatic sample injector and a photodiode-array detector system (Waters) was used for the separation and analysis of the phenylethanoid glycosides in *P. asiatica* L. extract. The separation was performed on a Shim-Pak C_{18} column (150×4.6 mm, 5 μm) with a C_{18} guard column (Shimadzu, Japan), and the column temperature was kept at 25 ℃ using a column heater-cooler. A binary mobile phase consisting of 0.2% acetic acid in water (A) and acetonitrile (B) was used for the separation. All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate of the pump was kept constant at 0.5 mL/min for a total run time of 30 min. The system was run with a gradient program: 20% B from 0—2 min, 20% B to 45% B from 2—20 min, and 45%B to 20% B from 20—30 min. The sample injection volume was 5 μL. The DAD was set at 320 nm.

The mass spectrometry experiment was performed on a Finnigan LCQ ion-trap mass spectrometer (MAT, San Jose, CA, USA) with an electrospray ion source. The LC system was connected to the mass spectrometer via a UV cell outlet. The mass spectrometer conditions were optimized for acteoside prior to sample analysis in order to achieve maximum sensitivity. As a result, the following mass spectrometer conditions were chosen: sheath gas flow rate 6×10^6 Pa, auxiliary gas flow rate 1×10^6 Pa, electrospray voltage of the ion source, 5 kV, capillary voltage −15 V, capillary temperature 270 ℃ and tube lens offset −20 V. Full scan of ions ranged from 50 to 1000 molecular weights in a negative ion mode. Source CID experiment was carried out to obtain detailed structural information about the phenylethanoid glycosides.

Results and discussion

The HPLC-DAD (HPLC Diode-Array Detector) chromatogram and LC-ESI-MS total ion chromatogram (TIC) of the crude extract from the aerial parts of *P. asiatica* L. are given in Figure 2. A good separation was

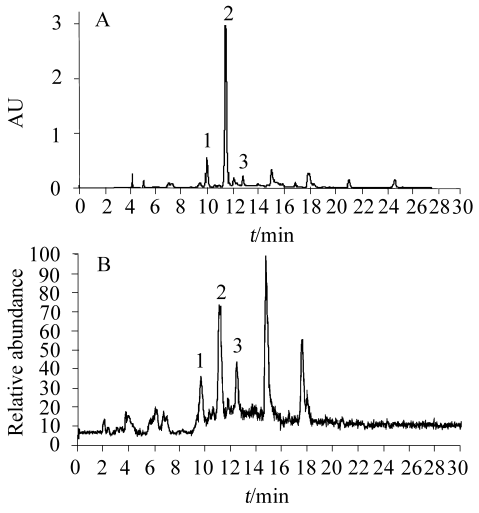


Figure 2 (A) LC-UV profile of *P. asiatica* L. crude extract. (B) LC-MS total ion chromtogram (TIC) of the extract of *P. asiatica* L. Compounds related to peaks 1—3 were identified as plantamajoside, acteoside and isoacteoside, respectively.

achieved within 30 min. Compounds related to peaks 2 and 3 were tentatively identified as acteoside and isoacteoside by congruent retention times (Table 1) with that of the authentic standards, while compound **1** was an unknown one.

Table 1 The LC-ESI-MS and corresponding CID data in the negative ion mode (*m/z* values) of peaks 1—3 in LC profile

Peak Identification	t_R[a]/min	M_r[b]	$[M-H]^-$ (*m/z*)[c]	CID (*m/z*)[d]
Plantamajoside (**1**)	9.62	640	639	477, 315, 179, 162, 135, 133
Acteoside (**2**)	11.08	624	623	477, 461, 315, 179, 162, 135, 133
Isoacteoside (**3**)	12.55	624	623	477, 461, 315, 179, 162, 135, 133

[a] Retention time. [b] Molecular weights. [c] Deprotonated molecular ion. [d] CID fragment.

To further investigate the structures of these three compounds, LC-ESI-MS and source CID experiments were attempted and the results are shown in Figures 3, 4, Table 1 and Scheme 1. Compounds related to the peaks in Figure 2 exhibited intense deprotonated molecular ions $[M-H]^-$ in the negative mode, from which the molecular weights of peaks 1—3 were confirmed to be 640, 624 and 624, respectively. As shown in Table 1, peaks 2 and 3 are isomers with the same molecular weight of 624.

The LC-ESI-MS of peak 2 is shown in Figure 3A. The deprotonated molecular ion $[M-H]^-$ (*m/z* 623) with high abundance was the only ion observed in the negative mode. Further investigation in the CID experiment of the *m/z* 623 ion ($[M-H]^-$) yielded 7 main fragment ions at *m/z* 477, 461, 315, 179, 161, 135 and 133 (Figure 3B), all of which were found to be produced directly from the parent ion (*m/z* 623). The ion at *m/z* 477 comes from the neutral loss of a rhamnose moiety (146), which is an external sugar unit in the disaccharide chain. The ion at *m/z* 461 is considered to be from the neutral loss of the caffeoyl moiety $[M-162-H]^-$ from the parent ion. The ion at *m/z* 623 loses the caffeoyl moiety and a rhamnose moiety to produce the *m/z* 315 ion as $[M-162-146-H]^-$. The ion at *m/z* 179 with a mass difference of 444 from the parent ion at *m/z* 623, corresponds to the loss of the aglycone moiety plus the disaccharide consisting of a glucose unit and a rhamnose unit. The *m/z* 161 ion is produced from the same pathway as the *m/z* 179 ion only with a further loss of one molecule of water. The ion at *m/z* 135 $[aglycone-18-H]^-$ arises from the cleavage of the glycosidic bond at C(1) position with an additional loses of one molecule water, with the charge remaining on the part of the aglycone moiety. The *m/z* 133 ion comes from the same pathway as the *m/z* 179 ion only with a further loss of two hydrogen atoms from *m/z* 135, and can be indicated as $[aglycone-18-2H-H]^-$. The ions at *m/z* 461, 315, 179, 161 and 135, obtained in our study

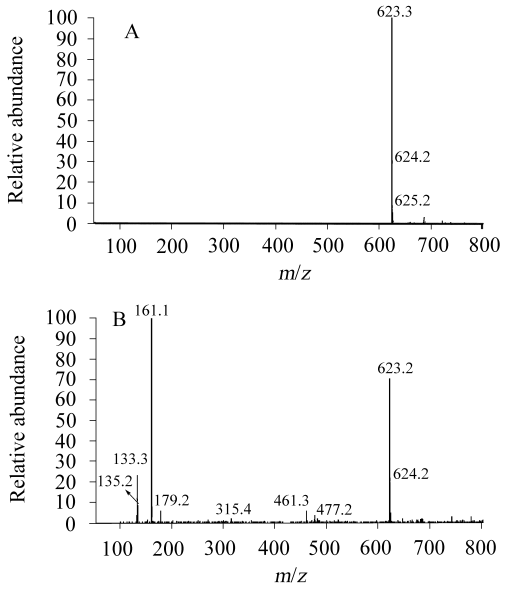


Figure 3 (A) LC-ESI-MS of peak 2 obtained in the negative ion mode. (B) The CID spectrum of the parent ion *m/z* 623 of peak 2. The related compound was identified as acteoside by its retention time and the CID pattern.

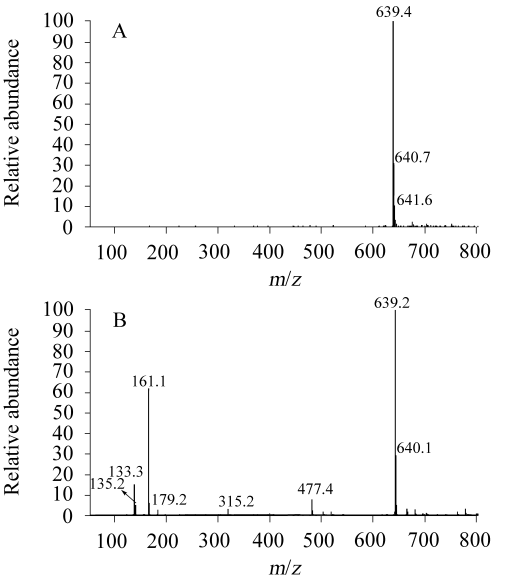
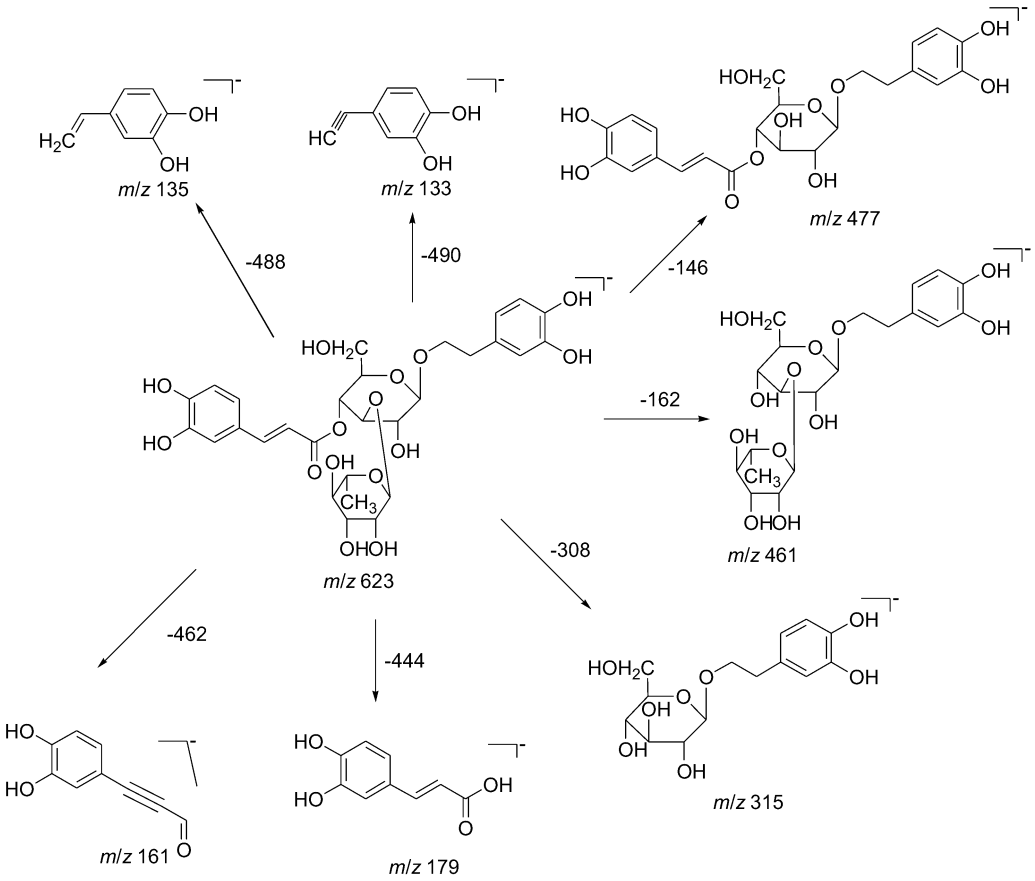


Figure 4 (A) LC-ESI-MS of peak 1 obtained in the negative ion mode. (B) The CID spectrum of the parent ion *m/z* 639. The related compound was identified as plantamajoside by its CID pattern and data from the literature (Refs. 3 and 4).

are consistent with the literature[3,4,19] report on acteoside from *Cistanches deserticola*.[19] However, the *m/z* 153 ion, which was assigned as $[aglycone-H]^-$ by Wang *et al.*,[19] was not found in our study. This ion may have been unstable and lose a molecule water to give *m/z* 135 in our experiment. In addition to the above ESI-MS/CID analyses, retention time of peak 2 were identical to that of acteoside, therefore, peak 2 was confirmed to be acteoside. Possible fragmentation pathways of peak 2 (acteoside) are illustrated in Scheme 1, which provides direct and detailed structural information about the ion at *m/z* 623.

Scheme 1 Proposed fragmentation pathway for the deprotonated molecular ion $[M-H]^-$ of acteoside



For peak 3, the LC-ESI-MS data showed *m/z* 623 as the deprotonated molecular ion $[M-H]^-$, an indication of the same molecular mass of 624 as peak 2 (acteoside). The CID experiment of peak 3 also produced the same pattern as peak 2, *i.e.* 7 fragmentation signals at *m/z* 477, 461, 315, 179, 161, 135 and 133 (Table 1), suggesting that these two compounds be possible isomers with similar structures. The compound related to peak 3 was therefore identified as isoacteoside by comparing its retention time with that of the standard, and by matching with reported mass spectral data.[19,20]

The LC-ESI-MS of peak 1 is shown in Figure 4A. A deprotonated molecular ion $[M-H]^-$ (*m/z* 639) is the only ion found in the negative mode. During CID experiment, the ion at *m/z* 639 formed 6 fragment ions: *m/z* 477, 315, 179, 161, 135 and 133 (Figure 4B). The ion at *m/z* 477 was produced directly from the parent ion of *m/z* 639, corresponding to the neutral loss of a caffeoyl moiety $[M-162-H]^-$. The ion at *m/z* 315 comes from the neutral loss of a caffeoyl moiety and a glucose moiety $[M-324-H]^-$. The other ions at *m/z* 179, 161, 135 and 133 in the CID spectrum followed the same fragmentation pathways as those of acteoside and isoacteoside as discussed earlier. According to the CID fragment patterns of these three compounds, and by comparing the structural information of acteoside and isoacteoside (peaks 2 and 3) and other similar phenylethanoids obtained in this and other studies,[3,4,19,20] we concluded that peak 1 was structurally highly related to acteoside. The only difference was the glucose unit at R^1 position in the disaccharide chain; it was glucose for peak 1 instead of rhamnose for peak 2 (acteoside). A tentative identity was therefore given to peak 1 as plantamajoside by using the information as discussed above and those in the literature. The proposed structure of plantamajoside is shown in Figure 1 (**1**). However, the exact structure of this compound needs to be confirmed by NMR spectroscopy and other analytical techniques in future work.

Conclusion

Although this LC-ESI-MS method was not subjected to full structural identification of the phytochemicals, it still has merits of being a simple and direct method for rapid identification of phenylethanoid glycosides in plant extract from *P. asiatica*. It eliminated tedious separation and purification procedures, and the retention time, molecular weights and the characteristic fragment ions of the known compounds together provided sufficient information for the confirmation of known compounds and at least for tentative identification of unknown compounds. Such a method is useful for samples that are known to contain certain phytochemicals, such as the phenylethanoid glycosides in *P. asiatica* L.

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