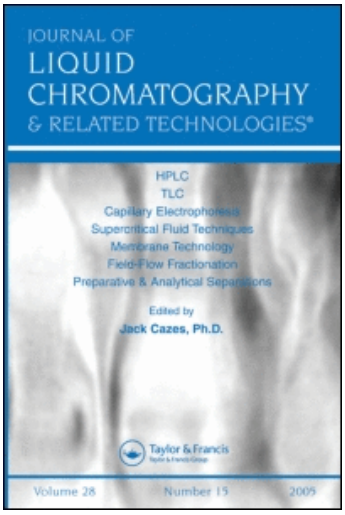

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Preparative Separation of Phenylpropanoid Glycoside from *Scrophularia ningpoensis* Hemsley by High Speed Countercurrent Chromatography and ESI-MS^n Analysis

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Abstract: The characteristic constituent angoroside C of the famous traditional Chinese medicine *Scrophularia ningpoensis* hemsley was separated from the crude extract by high speed countercurrent chromatography using reverse phase elution combined with silica gel column chromatography. Meanwhile, harpagoside was also obtained using the normal phase elution method. A two phase solvent system containing *n*-butanol:acetic acid:ethyl acetate:water (8:1:1:10) was selected according to the partition coefficient of angoroside C. A 311 mg quantity of the crude extract was loaded onto a 250 mL HSCCC column and yielded 49 mg angoroside C at over 89.1% purity, and 14 mg harpagoside at over 96.5% purity. Angoroside C was further purified by silica gel column chromatography and the purity reached 98.5% with high recovery. The chemical structures were determined by ESI/MS^n and ^{1}H-NMR. The characteristic ESI/MS^n fragmentation pattern of angoroside C and the proposed fragmentation mechanism are proposed.

Keywords: Angoroside C, Countercurrent chromatography, ESI-MS^n analysis, Preparative chromatography, *Scrophularia ningpoensis* Hemsley

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INTRODUCTION

The roots of *Scrophularia ningpoensis* Hemsl. are used as a famous Chinese medicine named "Xuanshen" for the treatment of inflammation, laryngitis, tonsillitis, abscesses of carbuncles, and constipation.[1] The plant is commonly used in combination with other herbs as a nutrient and a health strengthening agent.[2] Phenylpropanoid glycosides and iridoid glycosides are the two main kinds of chemical constituents in this plant and they play a key role in curing various kinds of diseases. These two kinds of glycosides are generally considered as the quality control marker for this plant.[3] Phenylpropanoid glycosides display quite interesting pharmacological properties including antioxidant, anti-viral, and inhibition of blood platelet aggregation, and LTB_4 synthesis.[4] Many phenylpropanoid glycosides including angoroside C (Figure 1), cistanoside D, acteoside, decaffeoylacteoside, have been isolated from plants of the genus *Scrophularia* (Scrophulariacaeae). Among those phenylpropanoid glycosides, angoroside C presents the highest content about 0.381% by weight in this plant.[5] The conventional methods for purification of angorosdie C are tedious and often require several steps.[6–7] Also, because of its phenolic hydroxyl groups, angoroside C has a tendency of being strongly adsorbed onto the solid support during separation.[8] In contrast, high speed countercurrent chromatography (HSCCC) has become an effective alternative to the conventional chromatographic techniques for the separation of some phenylpropanoid glycosides from plant extracts.[8–10] However, no report is available about separation of angoroside C from the plant extract by HSCCC.

In our previous studies, an efficient preparative separation method for the iridoid glycoside component harpagoside from *Scrophularia ningpoensis* Hemsl. was established.[11] During our following studies, the other remarkable constituent, angoroside C, for quality control of this plant was studied for its effective separation method. In this paper, we report an improved method about the application of HSCCC combined with silica gel column chromatography to separate the

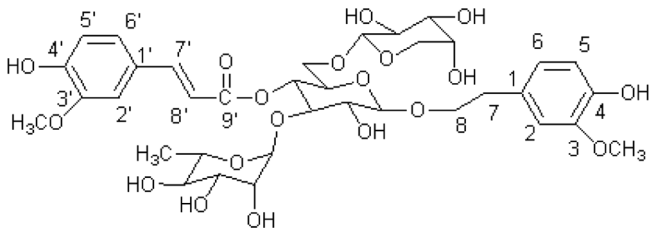

Figure 1. Chemical structure of angoroside C.

phenylpropanoid glycoside angoroside C from the crude extract of this plant. Two elution modes, including reverse phase elution and normal phase elution were used during separation of angoroside C and harpagoside in the HSCCC separation, because a large difference of polarity between the two compounds exists.

Characterization and analysis of angoroside C was accomplished by ESI-MS^n and ^{1}H-NMR experiments. ESI-MS^n analysis of phenylpropanoid glycosides including acteoside, isoacteoside, echinacoside, cistanoside A, acteoside, isoacteoside, and 2′-acetylacteoside have been reported.[9–10,12–14] However, no report was available about ESI-MS^n analysis of angoroside C. The characteristic fragment ions of angoroside C and the proposed fragmentation mechanism are presented and discussed in this paper.

EXPERIMENTAL

Apparatus

The countercurrent chromatography apparatus used in the present study is a TBE-300A multilayer coil planet centrifuge (made in 2006 by Shanghai Tauto Biotechnique, Shanghai, China), equipped with three preparative multilayer coils (column total volume 250 mL, wound with 1.6 mm I.D. PTFE tubing). The β values of this column range from 0.46 to 0.73 ($\beta = r/R$, $R = 6.5$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 rpm and 1000 rpm, where an optimum speed of 800 rpm was used in the present studies. The separation columns were installed in a vessel that was retained at 25°C by a Model HX-1050 constant temperature controller (Beijing Boyikang Lab Instrument Co., Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant flow pump (Beijing Shengyitong Technique Co., Beijing, China). Continuous monitoring of the effluent was achieved with a Model UV-II detector Monitor (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China) at 254 nm. SEPU3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-160 fractions collector (Shanghai Huxi Tech, Shanghai, China).

The HPLC system used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver.6.1 workstation.

Reagents and Materials

All organic solvents used for HSCCC were of analytical grade. Acetonitrile used for HPLC analysis was of chromatographic grade. The reagents were purchased from Hangzhou HuiPu Chemical Factory, Hangzhou, China.

Extraction of Crude Sample from *S. ningpoensis*

The roots of *S. ningpoensis* were collected in the south of Zhejiang province of China in September 2007, which was identified by Professor Qian Junqing, and a voucher specimen is deposited in the herbarium collection at Zhejiang University of Technology. A sample (200 g) of air dried root of *S. ningpoensis* was chopped and extracted 3 times, each for 1 h, with 90% ethanol under reflux. The ethanol extracts were combined and concentrated to dryness under reduced pressure to give a 20 g of crude extract. Water (400 mL) was added to the crude extract and the whole extracted successively with petroleum ether (250 mL × 3), ethyl acetate (250 mL × 3) and *n*-butanol (250 mL × 5) to yield 1.8 g petroleum ether extract, 3.2 g ethyl acetate extract, and 2.8 g *n*-butanol extract. The *n*-butanol extract was directly subjected to HSCCC.

Separation Procedure

The selected solvent system, consisting of *n*-butanol:acetic acid:ethyl acetate:water (8:1:1:10), was thoroughly equilibrated in a separation funnel by repeatedly vigorous shaking. The two phases were separated immediately prior to use. The multilayer coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow rate of 2.00 mL/min with the reverse phase elution mode, while the apparatus was run at a speed of 800 rpm. After hydrodynamic equilibrium had been reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (311 mg dissolved in 10 mL of a solution containing both the lower and upper phase (1:1, v/v) of the solvent system) was injected through the sample port. The reverse phase elution mode was stopped after angoroside C was eluted (0–270 min) and the normal phase elution was employed with the upper phase as the mobile phase (270–400 min). The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected in test tubes with a model BSZ-100 fraction collector (Shanghai Huxi Tech, Shanghai, China); each fraction was 6 mL. The

retention of the stationary phase was measured by collecting the total column content forced out with pressurized air.

Fractions, which contained angoroside C collected during the HSCCC separation, was further purified by open silica gel column chromatography (100 g of silica gel H, 100–200 mesh, Qingdao Haiyang Chemica, Qingdao, China), eluted by the mix solvent of ethyl acetate and 95% ethanol at the ratio of 5:1 to obtain fractions that contained angoroside C with high purity.

HPLC Analysis and Structure Identification

Each peak fraction obtained from HSCCC and silica column chromatography were analysed by analytical HPLC (Shimadzu, Kyoto, Japan). Column: Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm i.d.); the mobile phase: acetonitrile–water in linear gradient elution (acetonitrile: 0–20 min, 20–50%; 20–30 min, 50–90%, 30–60 min, 90%); flow rate: 0.6 mL/min; UV detector: 254 nm; the injection volume: 10 μL.

The chemical structure of angoroside C and harpagoside determined by ESI-MS^n was performed with a Therm LCQ^{TM} Deca XP plus ion trap mass spectrometry (Thermo Scientific, America) and a Bruker Avance 500 MHz spectrometer (Bruker Corporation, America) with TMS (tetramethylsilane) as the internal standard. A negative mode was used for data collection. The sheath gas and auxiliary flow rates were set at 96 and 7 (arbitrary unit), respectively. The capillary voltage was set at −29 V and its temperature was controlled at 350°C. The entrance lens voltage was fixed at 40 V and the multipole RF amplitude was set at 540 V. The ESI needle voltage was controlled at 4.5 kV. The tube lens offset was 16 V, the multipole lens 1 offset was 8.20 V and the multipole lens 2 offset was 10.5 V. The electron multiplier voltage was set at −980 V for ion detection.

RESULTS AND DISCUSSION

Selection of HSCCC Elution Mode

In CCC, either upper or lower phase can be chosen as the stationary phase, which is dependent on head-to-tail versus tail-to-head elution. Before deciding which phase is to be used as the stationary phase, the partition coefficient may be temporarily expressed as $K_{U/L} = C_U/C_L$, where C_U is the solute concentration in the upper phase and C_L, that of the lower phase. The two phase solvent systems with K values of the target compounds for HSCCC should be in the range of 0.5–1.0.[15]

The partition coefficient K is the ratio of solute distributed between the mutually equilibrated two solvent phases. Usually it is expressed by the amount of solute in the stationary phase divided by that of the mobile phase, as in conventional liquid chromatography. Totally, eleven two phase solvent systems were investigated for the two target compounds and the partition coefficient of both components was determined. The K values of angoroside C in the above solvent systems were measured by analytical HPLC according to the literature.[15] They are listed in Table 1. As shown in the Table 1, none of the following solvent systems was suitable for separation of both of the two components with the same elution method because both of K values of a solvent system can not meet the requirements. This can be explained by the major difference of polarity between angoroside C and harpagoside. Angoroside C owns a much bigger polarity than that of harpagoside due to their respective chemical structure. Two more strong hydrophilic moieties, including the rhamnose unit and arabinose unit exist in the molecule of angoroside C than that of harpagoside. The solvent systems *n*-butanol:acetic acid:water (4:1:5), *n*-butanol:water (1:1), *n*-butanol:ethyl acetate:water (4:1:5), and *n*-butanol:acetic acid:ethyl acetate:water (8:1:1:10) were suitable for separation of angoroside C when the upper phase was used as the stationary phase. Among those systems, the two systems *n*-butanol:acetic acid:water (4:1:5) and *n*-butanol:acetic acid:ethyl acetate:water (8:1:1:10) was possibly suitable for separation of harpagoside when the lower phase was used as the stationary phase with $1/K$ values 0.17 and 0.16.

Table 1. The K (partition coefficient) values of angoroside C and harpagoside in different solvent systems

	K ($1/K$) value	
Solvent system	Angoroside C	Harpagoside
Ethyl acetate:ethanol:water (50:1:50)	≈ 0 ($+\infty$)	0.12 (8.33)
Ethyl acetate:ethanol:water (10:1:10)	≈ 0 ($+\infty$)	0.53 (1.89)
Ethyl acetate:acetonitrile:water (2:1:2)	0.06 (16.67)	0.82 (1.22)
Ethyl acetate:ethanol:*n*-butanol:water (4.5:4.5:1:10)	≈ 0 ($+\infty$)	7.27 (0.14)
n-Butanol:acetic acid:water (4:1:5)	0.56 (1.79)	6.01 (0.17)
n-Butanol:water (1:1)	0.43 (2.33)	15.43 (0.06)
n-Butanol:ethyl acetate:water (4:1:5)	0.36 (2.78)	10.98 (0.09)
n-Butanol:ethyl acetate:water (3:2:5)	0.07 (14.29)	7.70 (0.13)
n-Butanol:ethyl acetate:water (1:4:5)	≈ 0 ($+\infty$)	1.42 (0.70)
n-Butanol:ethyl acetate:water (1:9:10)	≈ 0 ($+\infty$)	1.01 (0.99)
n-Butanol:acetic acid:ethyl acetate:water (8:1:1:10)	0.51 (1.96)	6.20 (0.16)

K: upper phase as the stationary phase.
$1/K$: lower phase as the stationary phase.

Therefore, two elution methods, including head-to-tail (upper phase as the stationary phase) and tail-to-head (lower phase as the stationary phase), were employed during the separation of angoroside C and harpagoside. The solvent system *n*-butanol:acetic acid:ethyl acetate:water (8:1:1:10) finally proved to be the best system for separation of angoroside C and harpagoside.

HSCCC Separation and HPLC Analysis

The typical HSCCC elution chromatogram detected at 254 nm is shown in Figure 2. Head-to-tail elution method, 0–270 min, (upper phase as the stationary phase); 270–400 min: tail-to-head elution method (lower phase as the stationary phase). Changing the above elution method can be easily achieved by directly changing the direction of rotation of the separation column without changing the inlet and outlet of the separation column. A 311 mg of crude extract was loaded onto the separation column in a single separation run. Angoroside C, 49 mg, and 14 mg harpagoside were separated. HPLC analysis showed that the purity of angoroside C reached 89.1% and the purity of harpagoside was about 96.5% purity. The crude sample was also analyzed by HPLC. Routine sample calculations were made by comparison of the peak area with that of the standard. The HPLC chromatogram was shown in Figure 3. The yield of angoroside C and harpagoside were 0.20% and 0.06%, respectively. The remainder of the injected crude extract, which remained in the tube of the coil has not been further investigated. The final retention of the stationary phase was 20.2%.

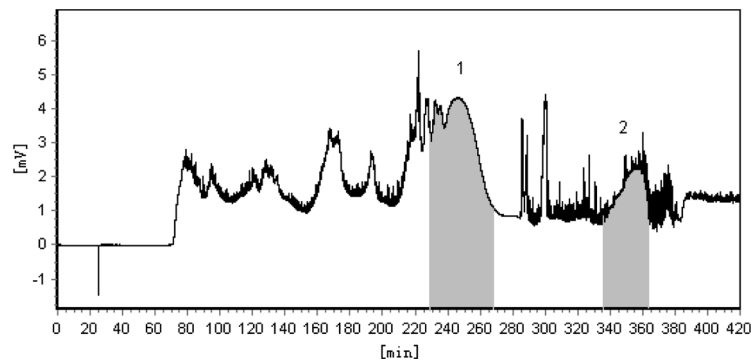

Figure 2. High-speed countercurrent chromatogram of a crude sample of *Scrophularia Ningpoensis* showing the separation of angoroside C (peak 1) and harpagoside (peak 2) from the matrix components. (For chromatographic protocol see Experimental section).

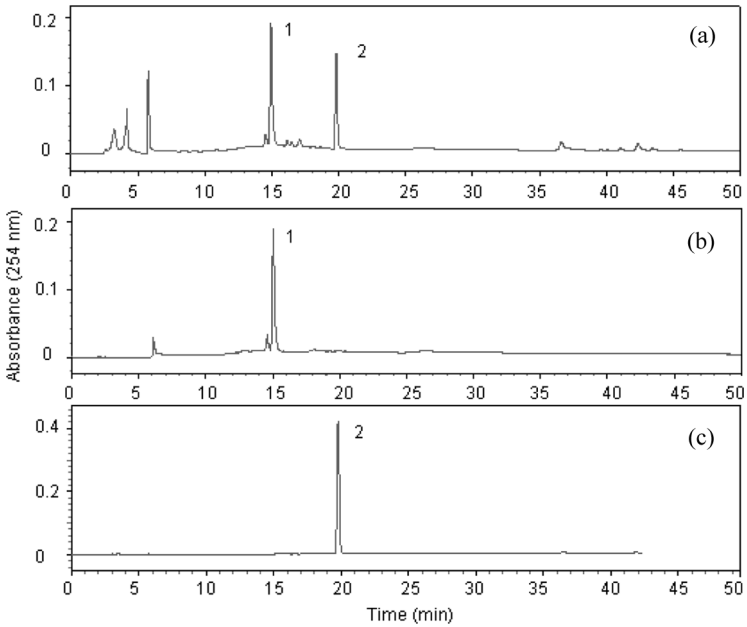

Figure 3. HPLC chromatogram of a crude sample of *Scrophularia Ningpoensis* and fraction 1 and 2 in HSCCC chromatogram. (For chromatographic protocol see Experimental section).

Silica Gel Chromatography Separation

The partially purified HSCCC fraction containing angoroside C weighed 49 mg with the purity of 89.1% was further purified by silica gel chromatography. Fractions that contained the target compound were collected and concentrated under reduced pressure, yielding 40 mg angoroside C with high purity over 98.5% determined by HPLC (HPLC chromatogram is not shown).

Comparison of the Traditional Separation Method and the Present One

Conventional isolation of angoroside C requires at least four separation steps with column chromatography including macro-porous resins using water-ethanol gradient elution, silica gel column chromatography with chloroform-methanol gradient elution, Sephadex LH-20 gel chromatography, and repeated silica gel chromatography again, all of which implies several days of labor in order to complete the separation work.[6] In comparison with this, a 311 mg quantity of an *n*-butanol extract was

separated by HSCCC and yielded 49 mg of angoroside C with the purity 89.1% in a separation run of only 5 h; and this partially purified fraction was further subjected to silica gel chromatography with isocratic elution to yield angoroside C with high purity at over 98.5%. Furthermore, none of the harmful solvent was used during the present separation process, while the very toxic solvent such as chloroform was needed in the traditional separation way.

Structure Identification

The ESI-MS and ^{1}H-NMR data for harpagoside was in agreement with what had been reported in our previous paper.[11] The structural identification of angoroside C was carried out by ^{1}H-NMR and negative ESI-MS^n spectroscopy. The data for the compound angoroside C were: ^{1}H-NMR (CD_3OD) δ ppm: 6.77 (1H, d, $J = 2.0$, H-2), 6.83 (1H, d, $J = 8.0$, H-5), 6.72 (1H, dd, $J = 8.0$, 2.0, H-6), 2.84 (2H, t, H-7), 7.22 (1H, d, $J = 2.0$, H-2′), 6.84 (1H, d, $J = 8.0$, H-5′), 7.09 (1H, dd, $J = 8.0$, 2.0, H-6′), 6.38 (1H, d, $J = 16.0$, H-8′), 7.69 (1H, d, $J = 16.0$, H-7′), 3.80, 3.85 (2×3 H, s, OCH_3), 4.40 (1H, d, $J = 7.9$, glu-1), 4.25 (1H, d, $J = 6.2$, ara-1), 5.21 (1H, brs, rha-1), 1.11 (3H, d, $J = 6.1$, rha-6). After comparing the data with ^{1}H-NMR spectral information from the literature,[6,7] the data was in agreement with that of angoroside C.

ESI-MS^n Analysis

Further identification of the structure of angoroside C by negative ESI-MS, MS^2 are shown in Figure 4. The ESI-MS of angoroside C in the negative mode gave m/z 783 as the intense deprotonated molecular ion $[M\text{-H}]^-$, which confirmed the molecular mass as 784, the same as that for angoroside C (Figure 4a). Further experiments in MS^2 of the m/z 783 ion ($[M\text{-H}]^-$) produced several main fragment ions at m/z 637, 607, 589, 475, 461, and 443 (Figure 4b). The ion at m/z 637 is considered to be from loss of the rhamnose unit $[M\text{-H-146}]^-$ from the parent ion m/z 783. The ion at m/z 607 is considered to be from loss of the caffeoyl moiety $[M\text{-H-176}]^-$ from the parent ion m/z 783; and m/z 589 was produced at the same time by losing one of neutral molecule H_2O $[M\text{-H-176–18}]^-$. The ion m/z 607 was further schizolysised to three main fragment ions at m/z 475, 461, and 443. The ion at m/z 475 is considered to be from loss of the arabinose unit from the daughter ion m/z 607 ($[M\text{-H-176–132}]^-$). The ion at m/z 461 is considered to be from loss of the rhamaose unit from the daughter ion m/z 607 ($[M\text{-H-176–146}]^-$); and m/z 443 was produced at the same time by losing one of neutral molecule H_2O

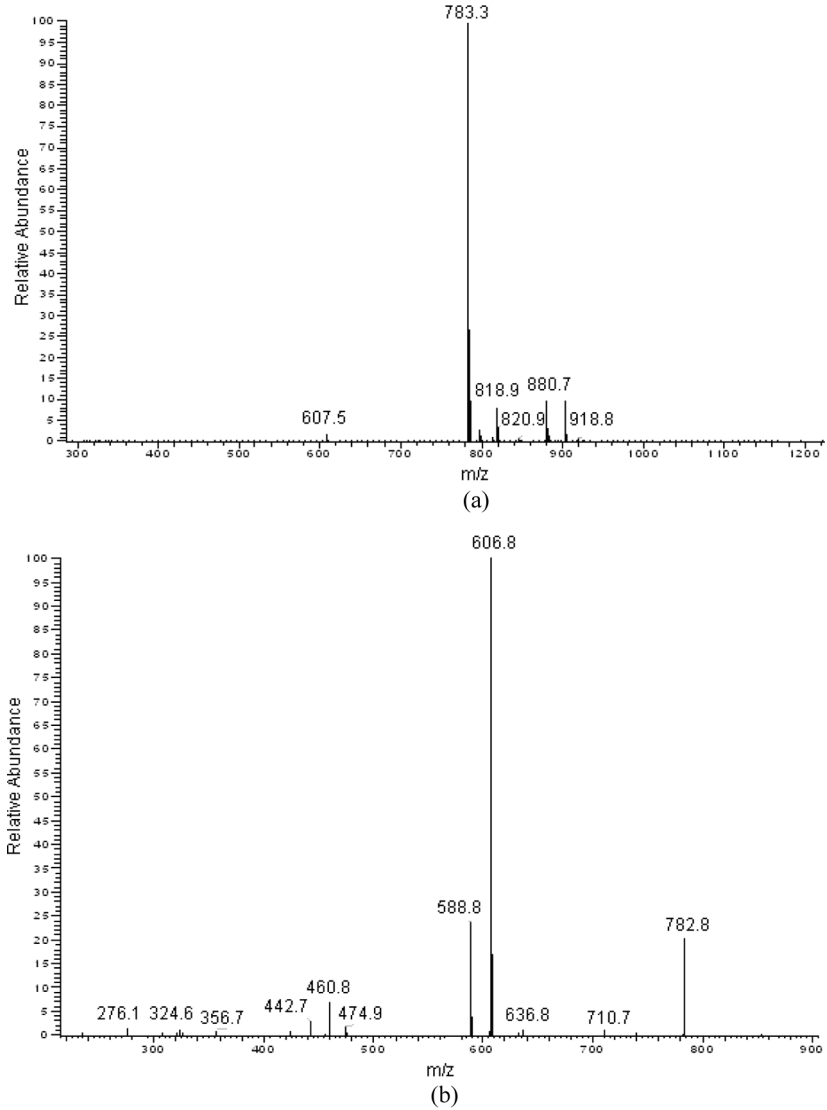

Figure 4. (a) ESI-MS fragmentation pattern of angoroside C. (b) ESI-MS^2 spectrum of m/z 783 of angoroside C.

([M-H-176-146-18]$^-$). The ESI-MS data provided highly useful structural information for the phenylpropanoid glycoside angoroside C, such as the neutral loss of the caffeoyl moiety (176), the rhamnose moiety (146), and the arabinose moiety (132). Possible fragmentation pathways of angoroside C is illustrated in Figure 5.

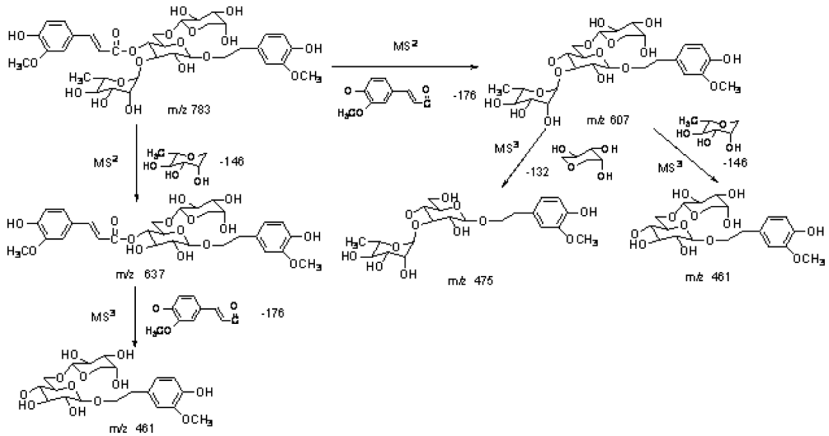

Figure 5. Proposed fragmentation pathway for angoroside C.

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