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Characterization and quantification of the triterpenoids in different parts of *Xanthoceras sorbifolia* by HPLC–ESI-MS

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ABSTRACT

Two new triterpenoid glycosides, sorbifoside A (**2**) and B (**3**), were isolated from the husks of *Xanthoceras sorbifolia* along with two known saponins (**1**, **5**). Their structures were established on the basis of 1D and 2D NMR data. A simple and sensitive assay was developed for the simultaneous determination of 6 triterpenoids (**1**–**6**, including two triterpenoid aglycones) in *X. sorbifolia* based on high performance liquid chromatography–mass spectrometry (HPLC–MS) coupled to an electrospray ionization (ESI) interface. The analytes were detected by positive ESI ionization mode and quantified by selected ion monitoring (SIM). All the linear regressions were acquired with $r^2 > 0.998$. The precisions were evaluated by intra- and inter-day tests, and the relative standard deviation (RSD) values were within the range of 2.0–2.8% and 1.7–2.9%, respectively. The recoveries for the quantified compounds were observed over the range of 95.3–104.7% with RSD values less than 4.6%. The method developed was successfully applied for simultaneous quantification of the six triterpenoids in *X. sorbifolia*, and our results showed that the contents of triterpenoids in different parts of *X. sorbifolia* varied significantly.

1. Introduction

The production and aggregation of the amyloid beta (Aβ) protein is widely believed to trigger the development of Alzheimer's disease (AD) [1]. During the last few years, we have focused our interest on the potential of yellowhorn (*Xanthoceras sorbifolia* Bunge, family Sapindaceae) against AD. Yellowhorn is a shrub native to China which has been recognized as a small ornamental tree and an important energy plant for bio-diesel [2]. In the past few decades, extensive areas of yellowhorn plantations have been established in northern China to combat desertification. We found that the crude extract of the husks of *X. sorbifolia* could significantly ameliorate the impairment of learning and memory in animal models induced by Aβ [3–6]. Phytochemical studies on this plant have resulted in the isolation and determination of a series of characteristic angeloyl (Ang)-substituted triterpenoids [7–15], among which xanthoceraside (**5**) was recently found to be the major determinant against Aβ-induced AD in mice [16,17]. Because of the structure complexity, our attempts to synthesize xanthoceraside had failed. Thus, naturally occurring xanthoceraside from yellowhorn has become a better way to solve the "supply problem" of the compound for further toxicity, pharmacokinetic and pharmacodynamic evaluations through *in vitro* and *in vivo* animal testing. Given the important ecological role of yellowhorn plantations in China, it is essential to obtain the compound as well as other angeloyl-substituted saponins from the regenerable parts of the plant, including fruit husks, seeds, flowers, leaves and small twigs.

However, there were no analytical methodologies available to simultaneously determine the contents of xanthoceraside (**5**) and other triterpenoids in this plant, nor is there any information on the distributions of the triterpenoids in different parts of the plant. As a result, twigs, seeds, husks, leaves and even the flowers have been used as folk medicines [18]. Thus, there has been an urgent need to propose a sensitive quantification method not only for determination of saponins in different parts of the plant but also for the quality control of yellowhorn-derived crude drugs.

In the present study, we developed an HPLC–ESI-MS based analytical method for simultaneous determination of six triterpenoids occurring in *X. sorbifolia* including two new saponins named as sorbifoside A (**2**) and B (**3**) characterized from the extract of husks.

2. Materials and methods

2.1. Chemicals

Methanol and formic acid (HPLC grade) were purchased from Fisher Scientific (USA). Deionized water was filtered through a

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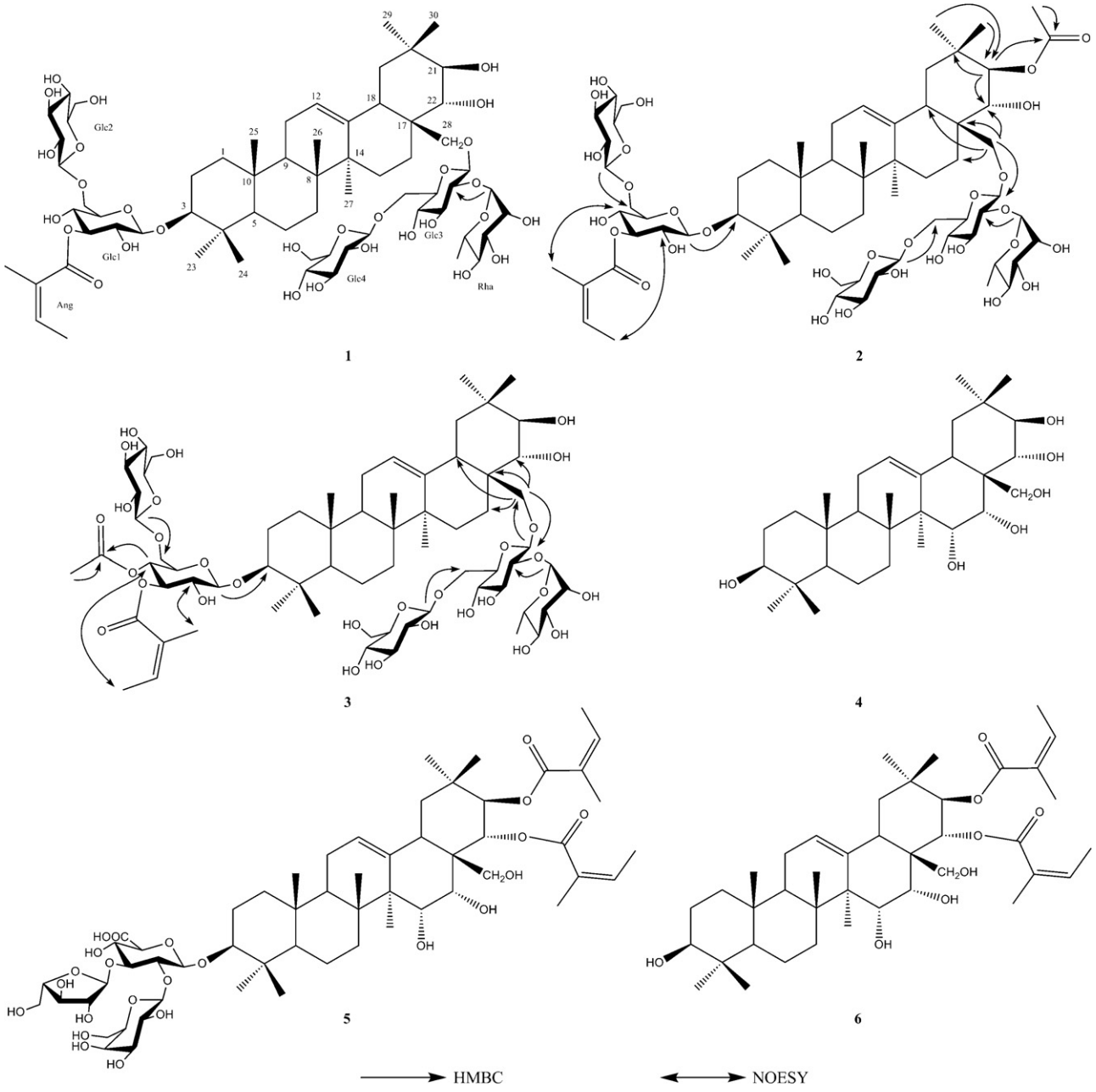

Fig. 1. Structures for compounds **1**–**6** and key 2D NMR correlations for the new compounds **2** and **3**.

0.22 μm filter before use. Other reagents and solvents were of analytical grade from Tianjin Damao Chemical Reagent Factory (Tianjin, China).

2.2. Plant materials and preparation of the triterpenoid analytes

Husks of *X. sorbifolia* for phytochemical studies were obtained from the Wudan Forest Farm in Wengniute County, Inner Mongolia, China. A voucher specimen (No. 0154620) was deposited in the Herbarium of Northeast China of the Institute of Applied Ecology, Chinese Academy of Sciences. Husks, twigs (~10 mm in diameter), leaves, flowers, and seeds for HPLC–MS analyses were collected at Shenyang Arboretum at Institute of Applied Ecology, Shenyang, China.

Dried husks (1 kg) of *X. sorbifolia* were extracted with 8 l (×3) of 70% ethanol under reflux for 2 h. The extracts were combined and concentrated to a small volume (~500 ml), and successively extracted with EtOAc (500 ml × 3) and n-BuOH (500 ml × 3). Evaporating of the solvents with a rotary evaporator under vacuum obtained 63.97 g of n-butanolic extract. Part of the extract (20.32 g) was subjected to vacuum flash chromatography over silica gel and eluted with gradient EtOAc–MeOH solvent system resulted in 8 fractions. Compounds **1**–**3**, **5** were purified from fraction 5 (EtOAc–MeOH = 6:4, v/v) using a semi-preparative HPLC system using an ODS-A YMC 10.0 × 250 mm column and eluting with 68% (v/v) methanol in water containing 0.05% trifluoroacetic acid (v/v) at a flow rate of 2.5 ml/min with UV detection at 220 nm. Compound **6** was prepared by acidic hydrolysis of **5** as described elsewhere [14]. Compound **4** was a gift from Dr. Zhanlin Li at School of Chinese Materia Medica, Shenyang Pharmaceutical University. All these compounds showed purities >98% by analytical HPLC.

Table 1
NMR data assignments for compounds **2** and **3**[a].

Position	**2**		**3**	
	δ_H	δ_C	δ_H	δ_C
1	1.70/1.03 m	38.9	1.59/1.01 m	38.9
2	2.36 dd (13.2, 2.7)/1.92 m	26.7	2.27/1.86 m	26.6
3	3.30 dd (11.7, 4.0)	89.5	3.30 dd (11.6, 4.3)	89.7
4	–	39.5	–	39.5
5	0.74 d (12.1)	55.8	0.71 d (11.9)	55.8
6	1.44/1.31 m	18.5	1.41/1.29 m	18.6
7	1.44/1.32 m	32.8	1.41 m	32.8
8	–	40.4	–	40.4
9	1.63 dd (11.0, 6.6)	48.0	1.60 m	48.0
10	–	36.9	–	36.9
11	2.11/1.96 m	24.1	1.93/1.85 m	24.0
12	5.51 t (3.3)	124.4	5.34 t (3.3)	123.7
13	–	142.3	–	143.1
14	–	41.8	–	42.1
15	1.68/1.08 m	25.7	1.89/1.22 m	26.0
16	2.10/1.97 m	18.8	2.31/1.93 m	18.3
17	–	43.5	–	43.4
18	2.83 dd (13.6, 3.3)	40.9	2.61 dd (13.6, 3.3)	41.8
19	2.15 t (13.6)/1.30 m	45.9	2.10/1.28 m	46.6
20	–	36.1	–	36.3
21	5.37 d (10.3)	79.5	3.73 d (9.8)	76.9
22	4.42 m	69.6	4.29 m	75.2
23	1.24 s	28.2	1.19 s	28.2
24	0.97 s	17.0	0.92 s	17.0
25	1.03 s	16.1	0.93 s	15.9
26	1.08 s	16.8	1.07 s	16.9
27	1.22 s	26.3	1.24 s	26.4
28	4.00 m	74.2	4.08 brs	76.0
29	1.02 s	29.7	1.21 s	30.5
30	1.15 s	20.4	1.25 s	19.7
Glc1				
1	4.86 d (7.7)	106.8	4.88 d (7.7)	106.5
2	3.98 m	73.5	4.03 m	73.1
3	5.90 t (9.5)	79.3	5.84 t (9.5)	76.1
4	4.27 m	69.6	5.45 t (9.8)	70.8
5	4.13 m	76.8	4.24 m	73.7
6	4.83/4.37 m	70.0	4.39 dd (11.0, 2.2)/4.09 m	69.2
Glc2				
1	5.09 d (7.6)	105.6	4.96 d (7.7)	105.0
2	4.04 m	75.2	4.01 m	75.2
3	4.21 m	78.4	4.20 m	78.5
4	4.21 m	71.7	4.20 m	71.7
5	3.91 m	78.4	3.90 m	78.5
6	4.51/4.40 m[b]	62.8[b]	4.48/4.32 m	62.8[b]
Glc3				
1	4.60 m	103.6	4.72 m	103.7
2	4.27 m	74.8	4.22 m	75.6
3	4.20 m	80.2	4.21 m	80.1
4	4.26 m	71.9	4.16 m	71.8
5	3.85 m	76.5	3.94 m	76.7
6	4.61/4.42 m	70.4	4.66/4.35 m	70.2
Rha				
1	6.54 (1H, brs)	100.6	6.51 m	101.0
2	4.66 (1H, m)	72.6	4.61 m	72.5
3	4.57 (1H, m)	72.5	4.71 m	72.5
4	4.29 (1H, m)	74.4	4.31 m	74.3
5	4.78 (1H, m)	68.8	4.79 dq (9.2, 6.0)	69.4
6	1.82 m	18.8	1.80 d (6.0)	19.0
Glc4				
1	5.02 m	105.8	5.00 d (7.8)	105.7
2	4.00 m	75.2	4.01 m	75.2
3	4.21 m	78.4	4.30 m	78.5
4	4.21 m	71.7	4.20 m	71.7
5	3.91 m	78.4	4.01 m	78.5
6	4.51/4.35 m[b]	62.7[b]	4.48/4.37 m	62.7[b]
Ang				
1	–	168.1	–	167.7
2	–	129.0	–	128.4
3	5.82 dq (6.9, 1.5)	137.0	5.86 dq (7.3, 1.1)	137.6
4	1.94 dd (6.9, 1.1)	16.0	1.93 dd (7.3, 1.1)	15.9
5	1.82 m	20.8	1.86 brs	20.7
Ac				
1	–	171.2	–	170.3
2	2.19 s	21.3	1.97 s	20.8

[a] ^{1}H and ^{13}C NMR were measured in pyridine-d_5 at 600 and 125 MHz, respectively. Multiplicities are indicated by usual symbols. Coupling constants (Hz) are in parentheses.
[b] Assignments with the same superscripts within the same column may be interchanged.

2.3. Structure elucidation

1D, 2D NMR spectra including ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, ^{1}H–^{1}H COSY and NOESY were acquired on a Bruker Avance 600 NMR spectrometer. A Bruker MicrOTOF-Q mass spectrometer was used for high resolution mass experiments. Structure elucidation was based on interpretation of NMR and mass spectroscopic data.

2.4. HPLC–ESI-MS analyses

Shimadzu 2010 series HPLC tandem mass spectrometer equipped with a LC-10AD*vp* binary pump, an on-line degasser, an auto-sampler and a column temperature controller were used for all LC–MS analyses. The data were processed using LCMSsolution ver. 3.0 (Shimadzu, Japan). Chromatographic separations were performed on a Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm; Dikma, Beijing, China) with a C_{18} guard column (12.5 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phase consisted of methanol (A) and 0.05% aqueous formic acid (B). The gradient program started with isocratic elution with 65:35 (A:B, v/v) for 25 min, then changed to 90:10 (A:B, v/v) in 10 min, and kept constant for 10 min. The flow rate was set at 0.8 ml/min with 30% of the eluent being splitted into the inlet of the mass spectrometer. Aliquots of 5 μl were injected into the HPLC system for analysis.

Mass data were acquired using a mono-quadrupole mass spectrometer coupled with an electrospray ionization source (ESI) in the positive ion mode with a capillary voltage of 1.5 kV, a curved desolvation line (CDL) temperature of 250 °C, a block temperature of 200 °C, an interface temperature of 400 °C, a heat block temperature of 200 °C, a CDL voltage of 10.0 kV and a detector voltage of 1.75 kV. Nitrogen was used as the sheath and auxiliary gas to assist nebulisation with the flow rate set at 1.5 ml/min.

Target ions were monitored $[M+2Na]^{2+}$ at *m*/*z* 698.40 for compound **1**, $[M+2Na]^{2+}$ at *m*/*z* 719.35 for compounds **2** and **3**, $[M+Na]^+$ at *m*/*z* 529.20 for compound **4**, $[M+Na]^+$ at *m*/*z* 1163.60 for compound **5** and $[M+Na]^+$ at *m*/*z* 693.40 for compound **6** using the selected ion monitoring (SIM) mode.

2.5. Calibration and quantification

Mixed standard stock solutions containing compounds **1**–**6** at the concentrations of 135.6, 67.20, 15.17, 34.80, 484.5 and 67.00 μg/ml, respectively, were prepared in methanol. Working standard solutions for the establishment of calibration curves were prepared by 80-, 40-, 20-, 8-, 4- and 2-fold dilutions of the mixed standard solutions with methanol and stored at 4 °C before use. Fresh plant samples including husks, twig bark, twig xylem, seed coats, seed kernels, flowers, and leaves, were freeze-dried and then ground with a home blender, and about 1.0 g of the powdered samples were extracted with 10 ml of 70% ethanol in an ultrasonic bath for 30 min (250 W, 40 kHz) at room temperature. The lost weight of the extracted solutions were compensated with 70% ethanol and filtrated through a 0.45 μm filter before injection. All samples were analyzed in triplicate.

Table 2
Linear regression, LOD, LOQ, precision, repeatability, and stability of 6 analytes.

Analyte	Regression equation[a]	r^2	Linear range (μg/ml)	LOD (ng/ml)	LOQ (ng/ml)	Precision (RSD, %)		Repeatability (RSD, %)	Stability (RSD, %)
						Intra-day	Inter-day		
1	$y = 1.652 \times 10^5 x + 5.865 \times 10^5$	0.998	1.695–67.80	17.21	56.50	2.3	2.7	3.0	1.8
2	$y = 9.71 \times 10^4 x + 1.487 \times 10^5$	0.999	0.840–33.60	19.06	70.03	2.7	2.0	2.7	2.7
3	$y = 2.953 \times 10^5 x + 2.325 \times 10^4$	0.999	0.1896–7.585	27.43	83.3	2.8	2.9	2.2	3.0
4	$y = 2.879 \times 10^5 x + 1.708 \times 10^5$	0.998	0.4350–17.40	22.61	85.9	2.0	2.7	2.6	2.6
5	$y = 1.179 \times 10^3 x + 4.253 \times 10^2$	0.998	6.056–242.3	2015	6043	2.2	1.9	3.0	2.9
6	$y = 1.000 \times 10^6 x + 3.447 \times 10^5$	0.999	0.838–33.50	15.04	45.67	2.2	1.7	1.4	3.0

[a] y is the peak area, x is the concentration injected.

3. Results and discussion

3.1. Structure elucidation

All the known compounds (**1**, **4**–**6**) were identified by MS, 1D and/or 2D NMR experiments and comparison with the published data [10,14,19].

Sorbifoside A (**2**), isolated as an amorphous white solid, gave a doubly charged $[M+2Na]^{2+}$ ion at m/z 719.3372 (calcd. for $C_{67}H_{108}O_{30}Na_2$, 1438.6721) by high resolution Q-TOF MS, thereby establishing the molecular formula of **2** as $C_{67}H_{108}O_{30}$. Compound **2** showed similar spectral features to those of the known compound **1** [19] except for the presence of an extra acetyl function resonanced at δ 2.19 (3H, s) in ^{1}H NMR spectrum and at δ 21.3, 171.2 in ^{13}C NMR spectrum, respectively. This was supported by the mass difference of 21 due to an acetyl group between their respective quasi-molecular ions of $[M+2Na]^{2+}$ in mass experiments. Another distinct difference was that the proton signal ascribed to H-21 was shifted downfield from δ 3.72 (1H, d, J = 9.8 Hz) to δ 5.37 (1H, d, J = 10.3 Hz), suggesting that **2** shared the same triterpenoid skeleton and sugar moieties with **1**, but the 21-hydroxy group have been acetylated. This was confirmed by the HMBC correlations between H-21 and the carbonyl carbon at δ 171.2. Furthermore, C-21 was shifted downfield, whereas C-22 was shifted upfield clearly. In 2D NMR experiments, the key correlations, such as Glc1 H-1 with C-3 (HMBC), Glc3 H-1 with C-28 (HMBC), Ang H-5 with Glc1 H-4 (NOESY), Ang H-4 with Glc H-2 (NOESY), are consistent with **2** being an acetate of **1** and having the structure shown in Fig. 1.

Sorbifoside B (**3**) was isolated as an amorphous white solid and produced doubly charged ions of $[M+2Na]^{2+}$ and $[M+Na+K]^{2+}$ at m/z 719.3359 and 727.3232 (calcd. for $C_{67}H_{108}O_{30}Na_2$ and $C_{67}H_{108}O_{30}NaK$, 1438.6721 and 1454.6460, respectively), respectively, in high resolution Q-TOF mass spectrum, corresponding to a molecular formula of $C_{67}H_{108}O_{30}$ which is identical with that of **2**. Similarly, an acetyl function could be found at δ 1.97 (3H, s) in ^{1}H NMR spectrum and at δ 20.8, 170.3 in ^{13}C NMR spectrum, respectively. However, the H-21 resonanced as a doublet at δ 3.73 (J = 9.8 Hz) implied an unsubstituted hydroxyl group at position 21. Compared with those of **1** [19] and **2**, the anomeric protons of Glc1 and Glc2 in the ^{1}H NMR spectrum of **3** were shifted downfield and upfield, respectively. Furthermore, all the signals due to Glc1 showed significant difference from those of **1** and **2**, but the upfield-shifted anomeric signals were the only clearly changed resonances in Glc2. Among all of these differences, the most striking distinction between **3** and **1** or **2** was the Glc1 H-4 which shifted from δ 4.27 to δ 5.45 (t, J = 9.7 Hz) in the ^{1}H NMR spectrum of **3**, indicating that the hydroxyl function at position 4 of Glc1 might have been acetylazed. This was confirmed by HMBC correlation found between Glc1 H-4 and the carbonyl carbon at δ 170.3 of the acetyl group. **3** was confidently determined as another acetate of **1** based on 2D NMR correlations shown in Fig. 1., and all the assignments of the NMR data were verified by intensive HSQC, HMBC, ^{1}H–^{1}H COSY and NOESY analysis (Table 1).

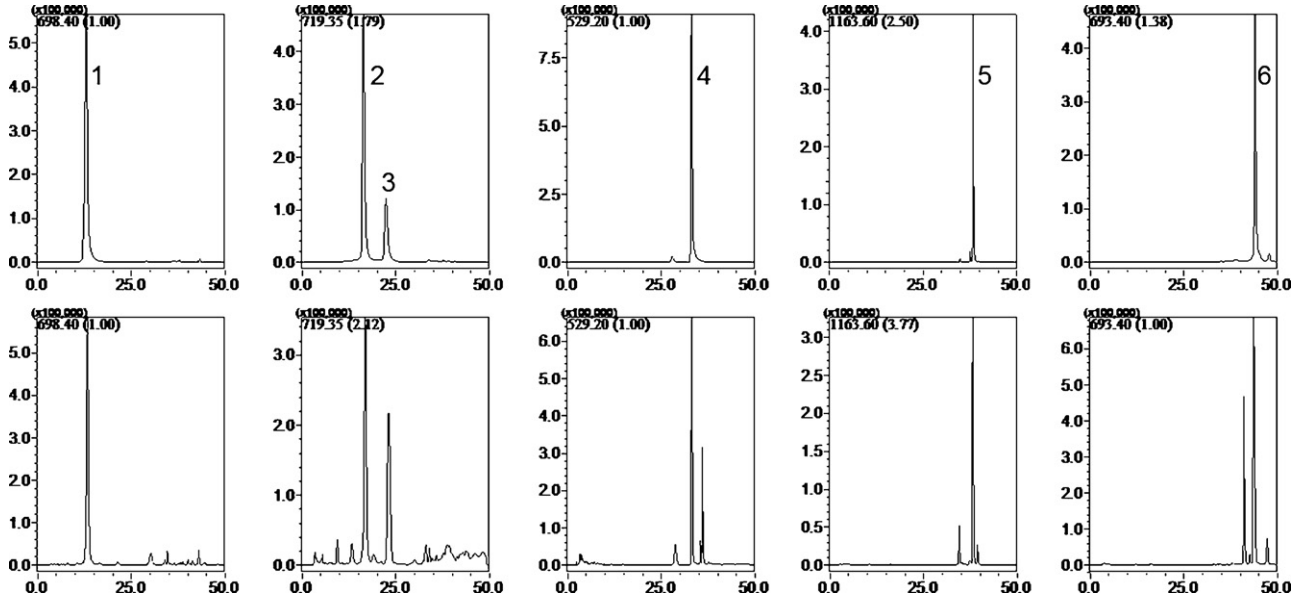

Fig. 2. Representative SIM chromatograms. Upper: mixed standards, lower: husks of *X. sorbifolia*. Selected ions corresponding to compounds **1**–**6** were monitored at m/z 698.40 (**1**, $[M+2Na]^{2+}$), m/z 719.35 (**2** and **3**, $[M+2Na]^{2+}$), m/z 529.20 (**4**, $[M+Na]^+$), m/z 1163.60 (**5**, $[M+Na]^+$) and m/z 693.40 (**6**, $[M+Na]^+$), respectively.

3.2. Optimization of extraction procedure

In order to obtain satisfactory extraction efficiency for all the analytes, extraction conditions, including extraction methods (ultrasonic and refluxing), extraction solvents (30%, 50%, 70%, 100% ethanol and methanol), and extraction time (15, 30, 45 and 60 min) were assessed based on single factor experiments. The best extraction efficiency was obtained by sonication extraction with 70% ethanol for 30 min.

3.3. Calibration curves, limits of detection and quantification

Standard stock solutions containing the 6 analytes were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the 6 analytes solutions were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas *versus* the concentration of each analyte. The calculated results were given in Table 2. All the analytes showed good linearity ($r^2 \geq 0.998$) over relatively wide concentration ranges.

The working solutions of the analytes were further diluted with methanol to yield a series of appropriate concentrations. The limits of detection (LOD) and the limits of quantification (LOQ) were established at signal-to-noise ratios of approximately 3 and 10, respectively. The LOD and LOQ were experimentally verified by injecting the 6 analytes at the LOD and LOQ concentrations. The results are shown in Table 2.

3.4. Precision, repeatability and stability

The intra- and inter-day precisions were determined by analyzing known concentrations of the 6 analytes in 6 replicates during a single day and by duplicating the experiments on 3 successive days. In order to confirm the repeatability of the developed assay, 6 different sample solutions prepared from the same sample were analyzed. Stability of sample solutions was analyzed at 0, 2, 4, 8, 12 and 24 h within 1 day at room temperature, respectively. Variations were expressed by relative standard deviations (RSD). As shown in Table 2, the RSD values of the intra- and inter-day precisions, repeatability and stability were all less than 3.0%.

3.5. Accuracy

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to approximately 0.50 g of the husk powder of *X. sorbifolia* samples. The resultant samples were then extracted and analyzed with the described method above. As could be seen from Table 3, the recoveries of the method was over the range 95.3–104.7% with RSD values less than 4.6%, indicating that the method was accurate for the determination of the six compounds in the husks of *X. sorbifolia*.

3.6. Sample analysis

The method was applied to simultaneously determine the 6 triterpenoids in different parts of *X. sorbifolia*, namely husks, twig bark, twig xylem, seed coats, seed kernels, flowers and leaves. Representative chromatograms are shown in Fig. 2. The contents of the 6 compounds in each sample were quantified and the results are shown in Table 4 with the mean values of three replicate injections.

Traditionally, the peeled twigs ("Wen Guan Mu" in Chinese) or the fresh leaves of yellowhorn have been used as a folk herbal medicine for treating rheumatic arthritis and gout. Phytochemical studies on the wood of *X. sorbifolia* have revealed the occurrence of flavonoids, tannins and unglycosylated triperpenes, of which flavonoids accounted for the largest amount of the methanolic extract of the wood [20–22], whereas dozens of saponins have been found from the fruits including seeds and husks [7–10,13–15]. The only compound purified and identified from leaves of yellowhorn was myricitrin [23]. Our results in Table 4 showed that husks contained all the six metabolites, five (**2**–**6**) of which were in their highest concentrations. Twig bark was found to be the second largest reservoir of the six metabolites in terms of their variety and abundance, where three out of the four detected compounds were in comparable contents with those in husks. Seed coats, seed kernels and leaves could only provided individual metabolites in very low concentrations. As the main active substance against AD, xanthoceraside (**5**) was the most abundant triterpenoid in all the plant parts except for the seed kernels and twig bark whereas new compounds **2** and **3** were found as minor triterpenoids present only in some parts of the plant. It is obvious that the husks were the best source for the potentially AD-suppressing triterpenoids. Furthermore, it will be a more sustainable way to use husks as the source of the active triterpenoids, because harvesting of the husks causes no hurt to the plants.

In conclusion, 6 triterpenoids were characterized from *X. sorbifolia* including two new compounds sorbifoside A (**2**) and sobifoside B (**3**). Our study also provided, for the first time, an HPLC–ESI-MS method for the simultaneous determination of the 6 compounds in *X. sorbifolia*. The developed analytical method was validated and applied to the real samples of *X. sorbifolia*, and thus provided an insight into the distributions of these metabolites in this plant.

Table 3
Recoveries of 6 analytes.

Analyte	Initial amount (μg)	Added amount (μg)	Detected amount (μg)	Recovery (%)	RSD (%)
1	215.3	113.0	329.5	101.1	1.9
		226.0	432.1	95.9	1.8
		339.0	538.2	95.3	2.4
2	68.60	33.60	102.8	101.8	4.6
		66.08	136.5	102.8	4.5
		106.4	173.8	98.9	2.8
3	30.90	15.74	46.92	101.8	3.4
		31.47	62.51	100.4	2.2
		44.96	74.10	96.1	1.8
4	58.60	29.00	87.8	100.7	1.9
		58.00	119.3	104.7	4.3
		83.5	143.6	101.8	3.6
5	814	408.0	1237	103.7	3.2
		816	1619	98.7	2.2
		1173	2037	104.3	3.3
6	77.80	40.20	118.8	102.0	2.4
		80.4	154.7	95.6	1.4
		115.9	197.4	103.2	4.1

Table 4
Contents (μg/g) of 6 analytes in different parts of *X. sorbifolia*.

Plant part	Analyte[a]					
	1	**2**	**3**	**4**	**5**	**6**
Husks	442.8	157.0	58.33	117.8	2392	120.6
Twig bark	662.6	9.84	30.14	–	2344	–
Twig xylem	341.5	tr	17.84	–	718.8	–
Seed coats	tr	–	–	–	33.80	tr
Seed kernels	tr	–	26.44	–	–	–
Flowers	tr	21.53	–	20.97	1919	2.079
Leaves	tr	–	–	–	458.5	tr

"–": below the LOD. "tr": below the linear range of calibration.
[a] The notation for analyte refers to Fig. 1.

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