Simultaneous Determination of Twenty-Two Components in Asari Radix et Rhizoma by Ultra Performance Liquid Chromatography Coupled with Quadrupole Time-of-Flight Mass Spectrometry

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Key words
- Asari Radix et Rhizoma
- Aristolochiaceae
- lignans
- essential oils
- aristolochic acids
- UHPLC-QTOF/MS

Abstract
Asari Radix et Rhizoma is a herbal medicine for the treatment of common cold, rhinitis, etc. An ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry method has been established for the rapid analysis of 22 components in 27 samples from the raw materials of Asari Radix et Rhizoma and an adulterant. A total of 5 lignans, 5 essential oils, 3 aristolochic acids, 4 alkamides, and 5 flavanoids were identified by co-chromatography of samples extracts and comparison of the retention time, UV spectra, characteristic molecular ions, and fragment ions with those of authentic standards, or tentatively identified by MS/MS determination along with MassFragment software. Moreover, the method was validated for the simultaneous quantification and semi-quantification of 20 components. The samples from Asarum heterotropoides var. mandshuricum differed in the quantity of 2-methoxyl-4,5-methylenedioxypropiophenone and kakuol from those of Asarum sieboldii var. seoulense, and the chemical difference was supported by principal component analysis and orthogonal partial least squared discriminant analysis based on dataset obtained from UHPLC-QTOF/MS. In comparison with the samples from the two medicinal Asarum species mentioned above, those from A. himalaicum differed in the quality and quantity of major compounds and contained higher amounts of aristolochic acid I.

Abbreviations
- AA: aristolochic acid
- AAN: aristolochic acid nephropathy
- ACN: acetonitrile
- AL-I: aristololactam I
- APCI: atmospheric pressure chemical ionization
- ARR: Asari Radix et Rhizoma
- DAD: diode-array detector
- ESI: electrospray ionization
- FWHM: full width at half maximum
- GAP: good agricultural practice
- LOD: limit of detection
- LOQ: limit of quantitation
- OPLS-DA: orthogonal partial least squared discriminant analysis
- PCA: principal component analysis
- PTFE: polytetrafluoroethylene
- QTOF/MS: quadrupole time-of-flight mass spectrometry
- RSD: relative standard deviations
- UHPLC: ultra high performance liquid chromatography
- XIC: extracted ion chromatogram

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Introduction
Asari Radix et Rhizoma (ARR, Xixin in Chinese) is a well known herbal medicine which has been mainly used to treat common cold, influenza, rhinitis, or as a local anesthetic agent, etc [1,2]. According to the Chinese Pharmacopoeia [3], it is derived botanically from the dry roots and rhizomes of Asarum heterotropoides Fr. Schmidt var. mandshuricum (Maxim.) Kitag., A. sieboldii Miq. var. seoulense Nakai., and A. sieboldii Miq.. Many pharmacological studies reported the extensive biological activities of ARR, including antimicrobial, antipyretic, anti-inflammatory, and analgesic properties [4–7]. Essential oils, lignans, and alkamides are the major components in ARR [8–10]
and should be responsible for the aforementioned activities [11]. Among the three Asarum spp. recorded under the same monograph of ARR in the Chinese Pharmacopoeia, the raw materials from A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense are called “Liaoxixin” in China, and both Asarum species are mixed in most herbal farms in northeastern China. The herbal materials from A. sieboldii have been rarely commercially available due to its depleted wild populations [12, 13]. Meanwhile, there are many adulterants of ARR in China, such as the roots and rhizomes of A. himalaicum Hook. f. et Thoms. ex. Klotzsch., A. caulescens Maxim., A. maximum Hemsl., and A. forbesii Maxim. [14]. Among them, the roots and rhizomes of A. himalaicum have been used as a folk medicine and are commercially available in a relatively large amount in some regions of western China [15]. However, there have been still no scientific studies to characterize the rationality of the traditional practice about ARR and its adulterants until now, which is a serious problem with regard to their efficacy, quality control, and safety [16].

According to the Chinese Pharmacopoeia [3], the essential oil and asararin are used as marker compounds for the quality control of ARR. Several holistic chemical profiling methods of ARR have been reported, such as quantification of 2-3 lignans and 1-2 constituents of essential oils using (HPLC)-UV [17–19]. It is worth noting that ARR contains AAs, some of which can cause AAN. The US FDA has prohibited the import of the herbal preparations in which the herbal medicines containing AAs are formulated [20]. Because ARR contains AAs in extremely small amount, an acceptable limit for AAs in ARR has been established in the Chinese and Japanese Pharmacopoeias. In the limit tests by HPLC method, the content of AA-I in dry materials of ARR must be less than 0.001% (10 ppm) according to the Chinese Pharmacopoeia [3], and the sample solution of ARR must show no peak at the retention time corresponding to AA-I from the standard solution in the Japanese Pharmacopoeia [21]. Although AA-I in ARR was determined by HPLC-UV and LC-APCI-MS/MS methods [22–24], few papers investigated the content of other AA compounds in ARR, such as AA-Iva and AL-I, in relation to AAN [25–27]. Thus, it is necessary to develop a method for simultaneous determination of active or marker components and AAs in ARR.

Generally, the use of conventional HPLC methods is difficult and time-consuming for simultaneous determination of lignans, essential oils, flavonoids, AAs, and alkaloids in ARR because of their relative low efficient stationary phases. Recently, the use of UHPLC coupled with QTOF/MS has been an effective approach for rapid analysis of multi-components of herbal medicines due to its higher peak capacity, greater resolution, increased sensitivity, and rich data on accurate molecular formulae for structural identification of unknown compounds [28, 29]. Since many unknown components may be present in ARR, most of which are new or without reference standards, using TOF/MS to identify them is important based on the molecular formula and fragmentation of the known components.

In the present study, a multi-component quantification fingerprinting approach based on UHPLC-QTOF/MS techniques has been developed, which combined the chemical profiling and semi-quantification of over 20 components in the roots and rhizomes of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense and the roots of A. himalaicum by UHPLC-QTOF/MS method.

Results and Discussion

In order to obtain satisfactory extraction efficiency for all the analytes, extractive methods (ultrasonic and refluxing), solvents (50%, 75%, and 100% methanol), and time (30 and 60 min) were assessed based on single factor experiments. The best extraction efficiency was obtained by refluxing extraction with 100% methanol for 30 min.

The methanolic extracts of A. heterotropoides var. mandshuricum (sample #3) and A. sieboldii var. seoulense (sample #10) were used for the optimization of UHPLC conditions; their representative UHPLC-UV and UHPLC-MS chromatograms are presented in Fig. 1. Using the optimal gradient elution as described in Material and Methods, all the specific peaks were satisfactorily separated within 22 min.

In order to characterize the chemical composition, the methanolic extracts of the root and rhizome of A. heterotropoides var. mandshuricum (sample #3) and A. sieboldii var. seoulense (sample #10) were subjected to UHPLC-QTOF/MS analysis. Twenty-two specific peaks (labeled peaks 1–22, Fig. 1) in the UHPLC chromatograms were characterized by UV absorptions obtained with Waters DAD. By co-chromatography and comparison of the retention time, UV spectra, and characteristic molecular ions and fragment ions with the authentic standards, compounds corresponding to 17 peaks were identified (Table 1).

Due to absence of reference compounds, the compounds corresponding to the rest 5 compounds were tentatively identified by MS/MS determination along with Waters MassFragment software, UV spectra, and comparison with literature data (Fig. 1 and Table 1). Peak 5 (tR 3.95 min) generated the positive molecular ion at m/z 597.1819 [M + H]+, corresponding to the molecular formula C27H32O15, and two fragment ions at m/z 435.1291 (loss of a hexosyl, 162 mass units) and 273.0763 (loss of two hexosyls, 324 mass units), which were the same as those of (2R)-naringenin 5,7-di-O-glucoside (peak 1, tR 1.78 min) in the MS chromatograms. The UV spectra of peak 5 (288, 323 nm) was also similar to that of (2R)-naringenin 5,7-di-O-glucoside (277, 320 nm), suggesting that it should be a flavanoid. Thus, the compound corresponding to peak 5 was tentatively identified as the isomer of (2R)-naringenin 5,7-di-O-glucoside. According to the UV spectra of peak 5, the compound corresponding to peak 5 may be (2R)-naringenin 4’,7-di-O-hexoside [30].

Peaks 7 (tR 6.53 min) and 9 (tR 7.52 min) generated the [M + H]+ ions at m/z 183.1021, corresponding to the same molecular formula C10H14O3. High collision energy resulted in two important fragment ions, at m/z 168.0779 (loss of a methyl, 15 mass units) and 153.0548 (loss of two methyls, 30 mass units). These fragment ions are in accordance with the fragmentation pathways of 3,4,5-trimethoxytoluene and 2,4,6-trimethoxytoluene shown in the phytochemical study on ARR [31]. According to the difference of polarity [32], peaks 7 and 9 were tentatively identified as 3,4,5-trimethoxytoluene and 2,4,6-trimethoxytoluene, respectively.

Peak 22 (tR 21.48 min) showed the similar UV absorption at 235 and 259 nm and generated the same [M + H]+ ion at m/z 248.014, corresponding to the molecular formula C12H14O2N. High collision energy resulted in diagnostic fragment ion at m/z 167.1310 (loss of C6H6, 81 mass units) and m/z 152.1075 (loss of C4H12, 95 mass units). The fragment ion was shown in the fragmentation pathways of a pair of isomers, N-isobutyl-2E,4E,8Z,10Z-dodecatetraenamide and N-isobutyl-2E,4E,8Z,10E-dodecatetraenamide in the studies by Yasuda et al. [10] and Luo et al. [33]. So the compound corresponding to peak 22 was tenta-
tively identified as isomer of N-isobutyl-2,4,8,10-dodecatetraenanamide.

Peak 18 (tR 17.30 min) generated the [M + H]+ ions at m/z 222.1858, corresponding to the same molecular formula, C14H23NO. High collision energy resulted in two diagnostic fragment ions, at m/z 167.1310 (loss of C4H7, 55 mass units) and m/z 152.1075 (loss of C5H10, 70 mass units). The same fragment ions were shown in MS data of peak 20 and 21, suggesting that the compound corresponding to peak 18 should also be an alkamide. According to the fragmentation pathways in the study by Quang et al. [8], the compound corresponding to peak 18 was tentatively identified as N-isobutyl-2E,4E,8Z-decatetraenamide (\(\text{Fig. 2}\)).

Good linear calibration curves were obtained with 18 tested reference standards (R > 0.995, \(\text{Table 2}\)). Because of a great difference in the contents of 2-methoxyl-4,5-methylene-dioxypropiophenone (8), kakuol (10), and methyleugenol (12) between the

Table 1  Marker compounds identified from A. heterotropoides var. mandshuricum, A. sieboldii var. seoulense, and A. himalaicum by UHPLC-QTOF/MS methods.

<table>
<thead>
<tr>
<th>Peaks No.</th>
<th>Compounds</th>
<th>t_R (min)</th>
<th>UV (nm)</th>
<th>[M + H]+ (m/z)</th>
<th>Aglycones or diagnostics fragments (m/z)</th>
<th>Comparison with standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2R)-naringenin 5,7-di-O-glucoside</td>
<td>1.78</td>
<td>277, 320</td>
<td>597.1819</td>
<td>435, 273</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>(2S)-naringenin 5,7-di-O-glucoside</td>
<td>1.99</td>
<td>277, 320</td>
<td>597.1819</td>
<td>435, 273</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>1-O-p-coumaroyl-xylopyranosyl-glucoside</td>
<td>2.00</td>
<td>232, 315</td>
<td>459.1503</td>
<td>481, 476, 165, 147</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>1-O-feruloyl-xylopyranosyl-glucoside</td>
<td>2.64</td>
<td>241, 330</td>
<td>489.1608</td>
<td>511, 506, 195, 177</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>isomer of (2R)-naringenin 5,7-di-O-glucoside</td>
<td>3.95</td>
<td>288, 323</td>
<td>597.1819</td>
<td>435, 273</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>AA-Iva</td>
<td>6.03</td>
<td>242, 330</td>
<td>358.0563</td>
<td>312, 297</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>3,4,5-trimethoxyltoluene</td>
<td>6.53</td>
<td>269, 300</td>
<td>183.1021</td>
<td>168, 153</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>2-methoxyl-methylenedioxypropiophenone</td>
<td>7.52</td>
<td>269, 332</td>
<td>209.0814</td>
<td>176, 161</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>2,4,6-trimethoxyltoluene</td>
<td>7.52</td>
<td>225, 280</td>
<td>183.1021</td>
<td>168, 153</td>
<td>no</td>
</tr>
<tr>
<td>10</td>
<td>kakuol</td>
<td>8.25</td>
<td>277, 346</td>
<td>195.0657</td>
<td>147</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
<td>pluviatilol</td>
<td>9.67</td>
<td>232, 285</td>
<td>357.1338</td>
<td>339, 289</td>
<td>yes</td>
</tr>
<tr>
<td>12</td>
<td>methyleugenol</td>
<td>10.83</td>
<td>231, 280</td>
<td>179.1072</td>
<td>164</td>
<td>yes</td>
</tr>
<tr>
<td>13</td>
<td>Al-I</td>
<td>10.92</td>
<td>239, 259</td>
<td>294.0766</td>
<td>n. d.</td>
<td>yes</td>
</tr>
<tr>
<td>14</td>
<td>AA-I</td>
<td>13.49</td>
<td>225, 244</td>
<td>342.0614</td>
<td>n. d.</td>
<td>yes</td>
</tr>
<tr>
<td>15</td>
<td>safrole</td>
<td>15.94</td>
<td>235, 286</td>
<td>163.0759</td>
<td>n. d.</td>
<td>yes</td>
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<tr>
<td>16</td>
<td>myristicin</td>
<td>16.67</td>
<td>239, 299</td>
<td>193.0865</td>
<td>192</td>
<td>yes</td>
</tr>
<tr>
<td>17</td>
<td>sesamin</td>
<td>16.98</td>
<td>241, 286</td>
<td>355.1182</td>
<td>337, 319, 289</td>
<td>yes</td>
</tr>
<tr>
<td>18</td>
<td>N-isobutyl-2E,4E,8Z-decatetraenamide</td>
<td>17.30</td>
<td>260</td>
<td>222.1858</td>
<td>167, 152</td>
<td>no</td>
</tr>
<tr>
<td>19</td>
<td>asarinin</td>
<td>18.09</td>
<td>241, 286</td>
<td>355.1182</td>
<td>337, 319</td>
<td>yes</td>
</tr>
<tr>
<td>20</td>
<td>N-isobutyl-2E,4E,8Z,10Z-decatetraenamide</td>
<td>20.45</td>
<td>235, 259</td>
<td>248.2014</td>
<td>167, 152</td>
<td>yes</td>
</tr>
<tr>
<td>21</td>
<td>N-isobutyl-2E,4E,8Z,10E-decatetraenamide</td>
<td>20.73</td>
<td>235, 259</td>
<td>248.2014</td>
<td>167, 152</td>
<td>yes</td>
</tr>
<tr>
<td>22</td>
<td>isomer of N-isobutyl-dodecatetraenamide</td>
<td>21.48</td>
<td>235, 259</td>
<td>248.2014</td>
<td>167, 152</td>
<td>no</td>
</tr>
</tbody>
</table>

n. d., not detectable
herbal materials, two calibrations were established to serve for the low and high content levels. The LODs and LOQs were in the range from 0.03 to 24.9 × 10⁻³ μg/mL and from 0.09 to 82.9 × 10⁻³ μg/mL, respectively.

The precision and recovery tests were done for 7 major marker compounds by the methods described in Materials and Methods. This method exhibited good reproducibility with intra- and inter-day variations (evaluated with RSD) of less than 4.8% (Table 25, Supporting Information). The recovery for these markers ranged from 99.0% to 103%, with RSD ranging from 2.0% to 5.8% (Table 25, Supporting Information). Thus, the analytical procedure is accurate and sufficiently sensitive for the simultaneous quantification of the major compounds in the raw materials of three Asarum species.

Using the UHPLC-QTOF/MS method, chemical profiling and quantification of the components from the roots and rhizomes of A. heterotropoides var. mandschuricum and A. sieboldii var. seoulense and the roots of A. himalaeicum were carried out (Fig. 1 and Table 3). There is a significant difference in the chemical profiling patterns among the roots and rhizomes of the three Asarum species.

Twenty-two components were commonly found in two raw materials of ARR (A. heterotropoides var. mandschuricum and A. sieboldii var. seoulense), including 5 lignans, 5 essential oils, 3 AAs, 4 alkaloids, and 5 flavonoids (Table 3).

Asarinin (19) (0.37–3.10 mg/g) and sesamin (17) (0.17–0.73 mg/g) were the major lignans in the samples of two Asarum species, asarinin (19) being a marker compound for the quality control of ARR according to the Chinese Pharmacopoeia. 2-methoxy-4,5-methylenedioxypropioophenone (8) and kakuol (10) were detected in much greater abundance in the samples of A. heterotropoides var. mandschuricum [0.49–1.22 mg/g for 2-methoxy-4,5-methylenedioxypropioophenone (8) and 0.11–0.25 mg/g for kakuol (10)] than in those of A. sieboldii var. seoulense [trace–0.07 mg/g for 2-methoxy-4,5-methylenedioxypropioophenone (8) and trace–0.01 mg/g for kakuol (10)] suggesting that both characteristic lignans might contribute to chemically distinguish the raw materials of the two Asarum species. Actually, the commercial samples of ARR mostly consist of A. heterotropoides var. mandschuricum and A. sieboldii var. seoulense due to their mixed plantation in most herbal farms. According to our results, there is not a relationship between the proportion of the mixed cultivation of different subspecies and the lignan contents. However, the difference in the lignan contents between Asarum heterotropoides var. mandschuricum and A. sieboldii var. seoulense is significant.

For example, kakuol (10) and 2-methoxy-4,5-methylenedioxypropioophenone (8) were detected in much greater abundance in the samples of A. heterotropoides var. mandschuricum than in those of A. sieboldii var. seoulense, which may serve for the chemical characterization of both Asarum herbal materials. Since the pharmacological studies reported the in vitro and in vivo antifungal activity of both lignans [34], they should be the candidates for standardization of herbal materials of ARR, and the mixed cultivation of the two Asarum plants might not be desirable for the quality control of raw materials of ARR. Zhang et al. [18] reported the determination of three major lignans, asarinin

![Figure 2](image_url)

**Figure 2** Fragmentation pattern and MS spectra of N-isobutyl-2E,4E,8Z,10E-dodecatetranamide (peak 21, a) and N-isobutyl-2E,4E,8Z-dodecatetranamide (peak 18, b).

### Table 2 Calibrations and detection limits for marker compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Calibration curve</th>
<th>r</th>
<th>Linear range (µg/mL)</th>
<th>LOD (× 10⁻³ µg/mL)</th>
<th>LOQ (× 10⁻³ µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2S)-naringenin 5,7-di-O-glucoside (1)</td>
<td>Y = 83.7x + 16.06</td>
<td>1.000</td>
<td>0.320–16.00</td>
<td>2.28</td>
<td>7.59</td>
</tr>
<tr>
<td>(2S)-naringenin 5,7-di-O-glucoside (2)</td>
<td>Y = 99.5x + 19.24</td>
<td>0.999</td>
<td>0.170–17.00</td>
<td>2.00</td>
<td>6.67</td>
</tr>
<tr>
<td>1-O-p-coumaroyl-xylopyranosyl-glucoside (3)</td>
<td>Y = 55.4x + 2.852</td>
<td>1.000</td>
<td>0.200–10.00</td>
<td>3.19</td>
<td>10.6</td>
</tr>
<tr>
<td>1-O-feruloyl-xylopyranosyl-glucoside (4)</td>
<td>Y = 256.6x + 42.71</td>
<td>0.998</td>
<td>0.050–4.000</td>
<td>0.37</td>
<td>1.25</td>
</tr>
<tr>
<td>AA-Va (6)</td>
<td>Y = 186.4x + 4.856</td>
<td>0.999</td>
<td>0.050–5.000</td>
<td>0.71</td>
<td>2.36</td>
</tr>
<tr>
<td>2-methoxy-4,5-methylenedioxypropioophenone (8)</td>
<td>Y = 2076x + 667.5</td>
<td>0.999</td>
<td>0.400–20.00</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Y = 1721x + 0.4944</td>
<td>0.998</td>
<td>0.004–0.400</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>kakuol (10)</td>
<td>Y = 818x + 94.4</td>
<td>0.998</td>
<td>0.200–10.00</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Y = 1057x + 3.834</td>
<td>0.999</td>
<td>0.002–0.200</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>pluviatilol (11)</td>
<td>Y = 339.7x–12.55</td>
<td>0.999</td>
<td>0.250–10.00</td>
<td>0.89</td>
<td>2.96</td>
</tr>
<tr>
<td>methyldeugenol (12)</td>
<td>Y = 64.80x + 17.19</td>
<td>0.997</td>
<td>5.000–100.00</td>
<td>0.72</td>
<td>2.41</td>
</tr>
<tr>
<td>Al-I (13)</td>
<td>Y = 54.29x + 1.018</td>
<td>0.998</td>
<td>0.100–5.000</td>
<td>0.12</td>
<td>0.39</td>
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<tr>
<td>AA-I (14)</td>
<td>Y = 3065x + 82.52</td>
<td>0.998</td>
<td>0.020–2.000</td>
<td>0.03</td>
<td>0.14</td>
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<tr>
<td>safrrole (15)</td>
<td>Y = 35.86x + 30.49</td>
<td>0.998</td>
<td>0.200–2.000</td>
<td>0.12</td>
<td>0.39</td>
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<tr>
<td>myristicin (16)</td>
<td>Y = 71.31x + 98.2</td>
<td>0.998</td>
<td>1.000–15.00</td>
<td>6.12</td>
<td>20.4</td>
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<tr>
<td>sesamin (17)</td>
<td>Y = 358.6x + 103.8</td>
<td>0.999</td>
<td>1.000–15.00</td>
<td>0.73</td>
<td>2.44</td>
</tr>
<tr>
<td>asarinin (19)</td>
<td>Y = 224.3x + 637.8</td>
<td>0.998</td>
<td>2.000–50.00</td>
<td>0.44</td>
<td>1.47</td>
</tr>
<tr>
<td>N-isobutyl-2E,4E,8Z,10E-dodecatetranamide (20)</td>
<td>Y = 118.8x + 186.5</td>
<td>0.999</td>
<td>5.000–100.00</td>
<td>0.26</td>
<td>0.88</td>
</tr>
<tr>
<td>N-isobutyl-2E,4E,8Z,10E-dodecatetranamide (21)</td>
<td>Y = 151.3x + 213.0</td>
<td>0.999</td>
<td>2.500–75.00</td>
<td>0.41</td>
<td>1.37</td>
</tr>
<tr>
<td>Isomer of N-isobutyl-dodecatetranamide (22)</td>
<td>Y = 31.6x + 5.444</td>
<td>0.999</td>
<td>0.630–50.00</td>
<td>0.46</td>
<td>1.53</td>
</tr>
</tbody>
</table>
Table 3  Content (mg/g) of 18 compounds in the roots and rhizomes of *A. heterotropoides* var. *mandshuricum*, *A. sieboldii* var. *seoulense* and the roots of *A. himalaicum*.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Flavanoids</th>
<th>AAs</th>
<th>Lignans</th>
<th>Essential oils</th>
<th>Alkamides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.87</td>
<td>0.56</td>
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</tr>
<tr>
<td>2</td>
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<td>0.94</td>
<td>0.51</td>
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</tr>
<tr>
<td>3</td>
<td>0.22</td>
<td>0.29</td>
<td>0.07</td>
<td>0.1</td>
<td>0.49</td>
</tr>
<tr>
<td>4</td>
<td>0.19</td>
<td>0.22</td>
<td>0.04</td>
<td>0.1</td>
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</tr>
<tr>
<td>5</td>
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<td>0.37</td>
<td>0.15</td>
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</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>0.24</td>
<td>0.07</td>
<td>0.1</td>
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</tr>
<tr>
<td>7</td>
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<td>0.16</td>
<td>0.05</td>
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<tr>
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<td>0.11</td>
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<td>0.35</td>
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<td>0.23</td>
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<td>0.05</td>
<td>0.1</td>
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<tr>
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<td>0.62</td>
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<td>0.31</td>
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<td>0.49</td>
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<tr>
<td>22</td>
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<td>0.07</td>
<td>0.07</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>1.28</td>
<td>n.d.</td>
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<td>0.49</td>
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<tr>
<td>27</td>
<td>1.44</td>
<td>1.67</td>
<td>n.d.</td>
<td>0.1</td>
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</tr>
<tr>
<td>28</td>
<td>0.11</td>
<td>0.07</td>
<td>n.d.</td>
<td>0.1</td>
<td>0.49</td>
</tr>
<tr>
<td>29</td>
<td>1.44</td>
<td>1.67</td>
<td>n.d.</td>
<td>0.1</td>
<td>0.49</td>
</tr>
</tbody>
</table>

a: The samples #1–#4 were botanically identified as *A. heterotropoides* var. *mandshuricum*, #5–#10 as *A. sieboldii* var. *seoulense*, the samples #26 and #27 as *A. himalaicum*. The rest samples (#11–#25, called "Liaoxixin" in China herbal market) were the raw materials from *A. heterotropoides* var. *mandshuricum* and *A. sieboldii* var. *seoulense*. n. d., not detectable (< limit of detection). tr., trace (> limit of detection but < minimum of the linear range). The numbers in bold represent the compounds as described in "Fig. 1; b: The contents of AA-I# 1–#4 are shown by the unit "ppm"
The chemical profiling of the essential oils of ARR was well studied using the GC-MS method [9]. In the present study, only 3 essential oils, methyl Eugenol (12), safrole (15), and myristicin (16) were detected and quantified by the UHPLC-UV or UHPLC-QTOF/MS methods. Safrole (15), possessing liver carcinogenic effects, was quantified by the UHPLC-UV method because it did not produce mass signal response due to its volatility. Methyl Eugenol (12), with central inhibition, antiinfectious and analgesic activity [35, 36], is a marker compound for the quality control of essential oils of the raw materials of ARR and the granules of ARR extract.

The limit test for AAs in ARR has been recorded in the Chinese and Japanese Pharmacopoeias [3, 21]. Three AAs in ARR were simultaneously quantified for the first time. AA-I (14) was found in trace amounts (less than 10 ppm) in all the samples of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense, but with a content of 6–8 ppm in those of A. himalaicum. AA-IVa (6) and AL-I (13) were detected in A. heterotropoides var. mandshuricum [0.07–0.16 mg/g for AA-IVa (6) and 0.03–0.07 mg/g for AL-I (13)] and A. sieboldii var. seoulense [0.03–0.04 mg/g for AA-IVa (6) and 0.03–0.04 mg/g for AL-I (13)]. Many papers reported the quantification of AAs in ARR. Sun et al. [37] reported that there is no or just a trace amount of AA-I (14) in the underground parts (namely roots and rhizomes) of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense in a small amount of AA-I (14) in their aerial parts. The results from the study of Chen et al. [38] showed that AA-IVa was detected in greater abundance than AA-I (14) in the A. heterotropoides var. mandshuricum roots and rhizomes (26.49–51.73 μg/g). According to our study, AA-IVa (6) and AL-I (13) were found in greater amount in A. heterotropoides var. mandshuricum than in A. sieboldii var. seoulense. Since AA-IVa (6) and AL-I (13) are the potential compounds for side effects of AAN, the planting of A. sieboldii var. seoulense should be preferable for GAP of ARR.

Four alkamides were simultaneously quantified or semi-quantified. Among them, N-isobutyl-2E,4E,8Z,10Z-dodecatetraenamide (20) and N-isobutyl-2E,4E,8Z,10E-dodecatetraenamide (21) were both major alkamides found in the samples of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense [1.32–4.37 mg/g for N-isobutyl-2E,4E,8Z,10Z-dodecatetraenamide (20) and 0.80–3.22 mg/g for N-isobutyl-2E,4E,8Z,10E-dodecatetraenamide (21)]. Some pharmacological studies indicated that both alkamides showed antioxidiant, immunostimulatory, and anti-inflammatory effects [39, 40]. So they should not be ignored in quality standardization of ARR.

Moreover, 5 flavonoids, (2R)-naringenin 5,7-di-O-β-D-glucoside (1), (2S)-naringenin 5,7-di-O-β-D-glucoside (2), 1-p-O-coumaroyl-xlyopyranosyl-glucoside (3), 1-O-feruloyl-xlyopyranosyl-glucoside (4), and the isomer of (2R)-naringenin 5,7-di-O-β-D-glucoside (5), were quantified and semi-quantified as minor components in the samples of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense as well the content of all of which were less than 1.0 mg/g.

The root of A. himalaicum is one of the adulterants of ARR in China. There is a significant difference in the chemical composition between the raw materials of A. heterotropoides var. mandshuricum and/or A. sieboldii var. seoulense, and those of A. himalaicum. Twelve components were detected in the roots of A. himalaicum, including 3 flavonoids, (2R)-naringenin 5,7-di-O-β-D-glucoside (1), (2S)-naringenin 5,7-di-O-β-D-glucoside (2), and the isomer of (2R)-naringenin 5,7-di-O-β-D-glucoside (5), 3 AAs, AA-IVa (6), AL-I (13), and AA-I (14), 3 lignans, 2-methoxyl-4,5-methylenedioxypropophenone (8), sesamin (17), and asarinin (19), 1 essential oil, safrole (15), and 2 alkamides, N-isobutyl-2E,4E,8Z,10Z-dodecatetraenamide (20) and N-isobutyl-2E,4E,8Z,10E-dodecatetraenamide (21). Among them, the 3 flavonoids were the major components of A. himalaicum. It is worth noting that AA-I (14) was detected in higher content in A. himalaicum than in the samples of two certified Asarum species in spite of AA-IVa (6) and AL-I (13) being found in similar amounts in the samples of three Asarum species. The results suggested that A. himalaicum should not be used as a medicinal substitute of ARR and should be prohibited in herbal markets in China.
16.97 min, m/z 353.1184, VIP 1.51), at the top right corner of “S”, are the marker compounds of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense, respectively, which contribute most to the difference between the raw materials of the two Asarum species.

The 7 leading markers between the raw materials of A. heterotropoides var. mandshuricum and/or A. sieboldii var. seoulense were structurally identified as N-isobutyl-(2E,4E,8Z)-decatrienamide (18) (a), kakuol (10) (b), 2-methoxy-4,5-methylene-dioxypipophenone (8) (c), 3,4,5-trimethoxyltoluene (7) (d), N-isobutyl-2E,4E,8Z,10E-dodecatetraenamide (21) (e), asarinin (19) (f), and sesamin (17) (g), respectively. The results of multivariate statistical analysis supported the quantification results in our study.

In conclusion, the present study is the first report on a UHPLC-QTOF/MS method for the rapid structural elucidation of 5 lignans, 5 essential oils, 3 AAs, 4 alkamides, and 5 flavanoids from the roots and rhizomes of A. heterotropoides var. mandshuricum, A. sieboldii var. seoulense and the roots of A. himalaicum. Moreover, the method was validated and applied for simultaneous quantification and semi-quantification of 20 compounds among them with good accuracy and precision. The difference in the chemical profiles among the samples of three Asarum species is helpful for the standardization and quality control of plant materials of ARR. Furthermore, the determined markers are representative according to multivariate statistical analysis.

Materials and Methods

**Chemicals and reagents**

Eighteen reference compounds were used in the present study (Fig. 15, Supporting Information). Methyleugenol (12), AL-1 (13), and AA-IVa (6), kakuol (10), safrole (15), sesamin (17) and asarinin (19) were from Shanghai Forever Biotech Co., Ltd. Other reference compounds used were isolated from the extracts of the roots and rhizomes of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense in our previous studies [41,42]. They included (2R)-naringenin 5,7-di-O-β-D-glucopyranoside (1), (2S)-naringenin 5,7-di-O-β-D-glucopyranoside (2), 1-O-p-coumaroyl-β-D-xlylopyranosyl-(1→6)-β-D-glucopyranoside (3), 1-O-feruloyl-β-D-xlylopyranosyl-(1→6)-β-D-glucopyranoside (4), 2-methoxy-4,5-methylene-dioxypipophenone (8), pluviatilol (11), and myristicin (16). The identity of these compounds was confirmed by melting point, UV, IR, 1H- and 13C-NMR, and MS, and their purities evaluated with HPLC-DAD were more than 98%. Two standards, N-isobutyl-2E,4E,8Z,10Z-dodecatetraenamide (20) and N-isobutyl-2E,4E,8Z,10E-dodecatetraenamide (21), were isolated from Echinacea purpurea in our previous study [43], and their structures were confirmed by melting point, UV, IR, 1H- and 13C-NMR, MS and compared with the literature. The standard of an isomer of N-isobutyl-2,4,8,10-dodecatetraenamide (22) was isolated from the extracts of the roots and rhizomes of A. heterotropoides var. mandshuricum and A. sieboldii var. seoulense, and its structure was tentatively identified by the UV spectra, MS/MS determination along with Waters MassFragment software, and comparison with literature [10]. However, the configurations of C2, C4, C8, and C10 could not be determined by MS/MS. The purities of the three compounds evaluated with HPLC-DAD were more than 98%. ACN (HPLC-MS grade) and formic acid (spectroscopy grade) were purchased from Fisher Scientific UK. Ultrapure water (18.2 MΩ) was daily prepared with a Milli-Q water purification system (Millipore). Leucine-enkephalin was obtained from Sigma–Aldrich.

**Plant materials**

Twenty-seven samples (samples #1 – #27) were directly obtained from China (Table 1S, Supporting Information). The plant materials were authenticated by Prof. Weining Wang (Liaoning Institute for Food and Drug Control, China). They were identified as A. heterotropoides var. mandshuricum or A. sieboldii var. seoulense according to the shape of the perianth lobes and leaves [44]. The perianth lobe of A. heterotropoides var. mandshuricum is erect with acuminate leaf blade apex, while that of A. sieboldii var. seoulense curls with acute leaf blade apex. We could only authenticate some samples according to the phytochemistry of the flowers in the fresh materials, while others from herbal markets were recorded as “Liaoxixing” according to their commercial
names and the general appearance identification. The voucher specimens are kept in the reference library for the medicinal herbs in Shenyang Pharmaceutical University. For voucher specimens numbers and collection details, see Table 15 Supporting Information.

Instrumentation and chromatographic conditions
UHPLC analysis was performed on a Waters Acquity HSS T3 column (2.1 × 100 mm, 1.8 µm, Waters) at 40°C. The mobile phase consisted of (A) water containing 0.2% formic acid and (B) acetonitrile containing 0.2% formic acid with gradient elution (linear gradient 9% B in 2.5 min, linear gradient 9% B to 31% B between 2.5 and 4 min, linear gradient 31% B between 4 and 15 min, linear gradient 31% B to 40% B between 15 and 16 min, linear gradient 40% B to 44% B between 16 and 17.5 min, linear gradient 40% B to 44% B between 16 and 17.5 min, finally linear gradient 44% B between 17.5 and 21.5 min). Re-equilibration duration (linear gradient 44% to 99% B between 21.5 min and 22 min, linear gradient 99% B between 22 and 23 min, linear gradient 99% B to 9% B between 23 and 23.1 min, finally 9% B between 23.1 and 25 min) was 2.5 min between individual runs. The flow rate was kept at 0.6 mL/min, and 2 µL of standard and sample solution were injected in each run.

Identification of marker compounds by UHPLC-QTOF/MS was performed on Waters QTOF Xevo G2 equipped with an ESI source, which gives a resolution of 10000 (FWHM) and mass accuracy error less than 5 ppm. Leucine-enkephalin was used as the lock mass to generate an [M + H]⁺ ion (m/z 556.2771) in the LockSpray mode at a concentration of 50 pg/µL at an infusion flow rate of 10 µL/min. The ESI source was operated in positive ionization mode with the capillary voltage at 3.0 kV, and the cone voltage was set to 25 V. Source and desolvation temperatures were set at 130 and 450°C, respectively. The nebulization gas flows were 800 L/h. All data collected in centroid mode were acquired using Masslynx™ NT 4.1 software (Waters Corp.).

Two different MS scanning experiments were used. (1) MS² experiment (E represents collision energy) uses an intelligent approach where parallel alternating scans are acquired both at low-collision and high-collision energy to obtain precursor ion information and full-scan mass fragment with precursor ion information in a single analytical run, respectively. The MS² experiment in two scan functions was carried out as follows. Function 1: m/z 100–1200, 0.2 s scan time, 6 V collision energy; and function 2: m/z 100–1200, 0.2 s scan time, collision energy ramp of 20–30 V. (2) MS/MS experiments were carried out by ramping collision energies from 20 and 30 V.

Preparation of standard solutions
Seventeen reference compounds, including (2R)-naringenin 5,7-dimethoxy-6-glucopyranoside (1), (2S)-naringenin 5,7-dimethoxy-6-glucopyranoside (2), 1-O-p-coumaroyl-β-D-xiloxyranosyl-(1→6)-β-D-glucopyranoside (3), 1-O-feruloyl-β-D-xiloxyranosyl-(1→6)-β-D-glucopyranoside (4), AA-Iva (6) 2-methoxy-4,5-methylenedioxypropophenone (8), kakkulol (10), pluviatol (11), methyleugenol (12), AL-I (13), AA-I (14), saffrole (15), myristicin (16), sesamin (17), asararin (19), N-isobutyl-2E,4E,8Z,10Z-dodecaetraenamide (20), and N-isobutyl-2E,4E,8Z,10E-dodecaetraenamide (21), as well as one tentatively identified compound, the isomer of N-isobutyl-2,4,8,10-dodecaetraenamide (22), were accurately weighed and dissolved in methanol to give individual stock solutions at suitable concentration. Series of working standard solutions were prepared by appropriate dilution of the stock solution with methanol in order to prepare calibrators. All solutions were stored at 4°C in refrigerator before analysis.

Sample preparation
Powdered herbal materials (0.5 g, passed through a 500 µm mesh sieve) were extracted by reflux with 50 mL of methanol for 30 min. The mixtures were centrifuged at 3000 rpm for 5 min, the supernatants were evaporated to dry under vacuum at 35°C, and the residues were dissolved with methanol, transformed to a 5 mL of volumetric flask and diluted with methanol to volume. An aliquot of each 2 µL filtrate filtered through a 0.22 µm PTFE syringe filter (Whatman, MN, Nas-gene, Advantec) was injected into the UHPLC instrument for analysis.

Method validation for quantification
Among 22 identified compounds, 18 compounds were quantified on an UHPLC-UV or UHPLC-QTOF/MS. Saffrole (16) was determined at the detective wavelength of 287 nm by UHPLC-UV method due to no response in the MS chromatogram. Twelve compounds (3, 4, 6, 8, 10–15, 17, and 19) were quantified using quasi-molecular ion chromatograms (XICs, with a 0.02 Da window), all peak areas of which were integrated at the expected retention times under full-scan MS conditions (Waters Quanlynx™ version 4.0 software). Five compounds (1, 2, and 20–22) were quantified using quasi-molecular ion chromatograms, all peak areas of which were integrated at the expected retention times under MS/MS conditions at m/z 435.1291 (1 and 2) and m/z 167.1310 (20–22). Due to lack of standards to complete the validation procedure, the calibration curves for peaks 5 and 18 were not established. Their contents were calculated by the method of semi-quantification with the calibrations of peaks 1 and 22, respectively, because peak 5 showed the same aglycone ion at m/z 435.1291 as peak 1, and peak 22 showed the same fragment ion at m/z 167.1310 as peak 18. A similar assumption was made by Liu et al. [45].

Calibration curves
Calibration curves (5-point) were obtained using external standard calibrations for 18 analytes injecting each solution in triplicates and then constructed by plotting the peak area versus the concentration of each analyte.

Limit of detection and of quantitation
The stock solutions of 18 reference compounds were diluted to a range from 0.03 to 24.9 × 10⁻⁴ µg/mL, and the injection volume was 2 µL. LOD and LOQ were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Precision, accuracy, repeatability, stability, and recovery
The intra- and inter-day precisions were evaluated by analyzing known concentrations of the 7 analytes, including 1-O-p-coumaroyl-β-D-xiloxyranosyl-(1→6)-β-D-glucopyranoside (3), 2-methoxy-4,5-methylenedioxypropophenone (8), methyleugenol (12), AL-I (13), saffrole (16), asararin (19), and N-isobutyl-2E,4E,8Z,10E-dodecaetraenamide (21), in six replicates during a single day and by duplicating the experiments on 3 successive days. Six different sample solutions prepared from the same sample were analyzed to confirm the repeatability of the developed assay. Stability of sample solutions was analyzed at 0, 2, 4, 8, 12 and 24 h at room temperature, respectively. Variations were expressed by RSD.
The recovery was used to evaluate the accuracy of the method. A known amount of the 7 standards mixed solutions were added into a certain amount of the samples of “Liaoxinlin” (A. heterotropoides var. mandschuricum and A. sieboldii var. soulense) (0.25 g, sample #17). The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test.

Chemometric data analysis
The UHPLC-MS data of A. heterotropoides var. mandschuricum and A. sieboldii var. soulense samples were analyzed by MarkerLynx XS software (Waters). The parameters were set as following: retention time range 1.5–22.0 min; mass range m/z 100–600 Da; retention time tolerance 0.1 min; mass tolerance 0.05 Da; width of an average peak at 5% height and peak-to-peak baseline noise was automatically calculated; marker intensity threshold 10.0; noise elimination level 6.0; isotopic peaks were excluded for analysis.

Supporting information
The chemical structures of the analysed compounds, collection data of the herbal material samples used in the study, as well as intra-day, inter-day precision and recovery of the seven major marker compounds are available as Supporting Information.

Acknowledgments
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Conflict of Interest
There are no financial/commercial conflicts of interest involving any of the authors of this study.

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