Original Article

Rapid quantitative analysis of 12 chemical constituents in wild-simulated and cultivated *Astragali Radix* based on UHPLC-MS

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A R T I C L E   I N F O

A B S T R A C T

Objective: Astragali Radix (AR) is one of the most widely used traditional Chinese medicines (TCMs) for tonic, which can be divided into wild-simulated and cultivated AR according to its cultivation method. However, whether cultivated AR can replace wild-simulated AR has always been a concern.
Methods: In this study, a rapid, highly sensitive and specific analytical method using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS) was developed to quantitatively measure 12 chemical constituents of AR in the different cultivation methods.
Results: AR samples were analyzed with a good linear regression relationship (R^2, 0.9983–0.9995), precisions (relative standard deviation (RSD), 1.31%–2.36%), repeatability (RSD, 2.65%–4.92%), stability (RSD, 1.50%–4.05%), and recovery (95.13%–106.52%). Through the determination of AR samples, we found the components of flavonoids in wild-simulated AR were higher than cultivated AR, the saponins in cultivated AR were higher, the ratio of saponins/flavonoids in cultivated AR was higher than wild-simulated AR.
Conclusion: Based on this research, it could provide guidance for the quality control of AR.

1. Introduction

Astragali Radix (AR) is the dried roots of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao (*A. mongholicus*) or *A. membranaceus* (Fisch.) Bge. (*A. membranaceus*) (Wang et al., 2020). Clinical researches showed that AR exerted a wide range of medicinal values, such as cardiovascular disease (Liu et al., 2020; Kong et al., 2021), immunomodulation (Chen et al., 2020; Wei et al., 2021), anti-hyperglycemia (Mao et al., 2009; Zhang et al., 2020), anti-inflammatory (Ryu et al., 2008; Lai, 2018), antioxidant (Lin et al., 2019; Song et al., 2020), and antiviral activities (Chen et al., 2020; Ko, Wei, & Chiou, 2006). Modern researchers have found that the main constitutes in AR are saponins, flavonoid glycosides and their corresponding aglycone, and polysaccharides (Mei et al., 2020; Xue, Li, Li, Liu, & Qin, 2019; Yin et al., 2018). AR could be divided into wild-simulated AR and cultivated AR according to their cultivation method. The growth period of wild-simulated AR is at least six years and its roots grow downward, which is difficult to dig than cultivated AR. The culti-

vated AR is to raise seedlings for one year, then transplant horizontally with short growth period and easy harvest (Qin et al., 2013). Therefore, the cultivated AR is considered to be a medicinal material developed to solve the shortage of supply of wild-simulated AR. However, whether cultivated AR can completely replace wild-simulated AR is a worthy question. As we all know, the research on the chemical components of traditional Chinese medicines (TCMs) is the prerequisite for clarifying its effective substances, pharmacological effects, mechanism, and clinical efficacy (Hou et al., 2019; Deng et al., 2019; Liu & Xie, 2017). Clarifying the chemical composition of AR could provide a reliable theoretical basis for the targeted development of AR products. Therefore, it is of great significance to compare the chemical differences between these two styles of AR.

Liquid chromatography-mass spectrometry (LC-MS) originated in the 1970s and is currently-one of the most widely used analytical methods, which combines the high separation performance of chromatography with the high discrimination ability of mass spectrometry to achieve the complementary advantages of the two. For the analysis of TCMs, the LC-MS has the advantages of high efficiency, rapidity, high sensitivity, simple sample processing, and low dosage. It has demonstrated its advantages in chemical

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composition analysis, quality control, structure identification, isomer discrimination, pharmacokinetics of TCMs (Li et al., 2020).

In this experiment, an analytical efficient strategy using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used to analyze the contents of 12 saponins and flavonoids in wild-simulated and cultivated AR.

2. Materials and methods

2.1. Materials and reagents

The wild-simulated AR was purchased from Shanxi Datong Hunyuan Wansheng Astragalus Development Co., ltd. (Datong, China). The cultivated AR was collected in Yingxian, Shanxi Province, China. All of them were authenticated by Prof. Xuemei Qin of the Modern Research Center of Traditional Chinese Medicine of Shanxi University. Standard reference compounds calycosin (**1**) was purchased from Chengdu Must Bio-technology Co., ltd. (Chengdu, China). Other reference compounds, including calycosin-7-O-β-D-glycoside (**2**), onion (**4**), (6aR, 11aR) 9,10-dimethoxyisoflavane-glucoside (**5**), (6aR, 11aR) 9,10-dimethoxyptercarpan (**6**), 7,2′-dihydroxy-3′,4′-dimethoxy isoflavan (**7**), 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-O-β-D-glucoside (**8**), astragaloside I (**9**), astragaloside III (**11**) and astragaloside IV (**12**) were purchased from Jiangsu Yongjian Pharmaceutical Technology Co., ltd. (Nanjing, China). Formononetin (**3**) and astragaloside II (**10**) were purchased from Shanghai Eternal Biotechnology Co., ltd. (Shanghai, China). The purity of these reference compounds was over 98% according to HPLC analysis, and their chemical structures were shown in Fig. 1.

Formic acid and acetonitrile of HPLC grade were bought from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Deionized water used throughout the experiment was purified by a Milli-Q Gradient Å10 System (Millipore, Billerica, MA, USA). Other used reagents were of analytical grade in this study.

2.2. Preparation of AR

AR were soaked with 10 times of distilled water for 30 min, and then refluxed and extracted for 2 h. After filtered, the extraction residue was re-extracted with 8 times of distilled water for 1.5 h. The two extracted solution were combined, concentrated and evaporated to obtain a concentrated solution. Finally, freeze-dry

the prepared concentrated solution to obtain AR powder. The yield of wild-simulated AR was 24.28%, while the cultivated AR was 25.78%.

2.3. Sample preparation

The wild-simulate and cultivated AR samples (Positive ion mode: 29 mg, Negative ion mode: 200 mg) were added with 1 mL of methanol, vortexed for 1 min, ultrasonically extracted for 20 min, respectively. The extracts were centrifuged at 13000 rpm for 15 min (4 °C). The supernatant was filtered through 0.22 μm microporous membrane.

2.4. Preparation of standard stock solutions

Standard reference solutions of 12 analytes were firstly prepared by dissolving a precisely weighted portion of the standards in methanol. Standard stock solutions **A** and **B** consisted of six analytes (**1–6**) and six analytes (**7–12**) were prepared through mixing appropriate amount of standard stock solution, which consisted of the final concentration of each analyte as follows: 21.6 μg/mL (**1**), 38.064 μg/mL (**2**), 11.88 μg/mL (**3**), 17.44 μg/mL (**4**), 17.41 μg/mL (**5**), 6.427 μg/mL (**6**), 15.5 μg/mL (**7**), 46.8 μg/mL (**8**), 214 μg/mL (**9**), 61.2 μg/mL (**10**), 28.2 μg/mL (**11**) and 95.5 μg/mL (**12**). All those solutions were analyzed in positive and negative mode, respectively. Then, the working standard solutions were prepared by diluting the stock solutions with methanol to a series of proper concentrations. All standard solutions were stored in brown vials at 4 °C and filtered through 0.22 μm membrane filters before analysis.

2.5. Chromatography and mass spectrometry analysis

The separation was performed on an Agilent 1290 Infinity II UHPLC system (Agilent, Santa Clara, CA, USA) consisting of a quaternary solvent delivery system. And the chromatographic separation was achieved on a Waters ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm, Waters, Milford, MA, USA). The mobile phase consisted of solvent A: 0.2% aqueous formic acid and solvent B: acetonitrile using a linear gradient, which was programmed as follows: 0–5 min, 1%–20% B, 5–8 min, 20%–25% B, 8–15 min, 25%–30% B, 15–18 min, 30%–40% B, 18–20 min, 40%–60% B, 20–23 min, 60%–99% B, 23–25 min, 99%–1% B. The flow rate was set at 0.25 mL/min and the sample injection volume was 5 μL. The column temperature was conditioned at 45 °C.

An AB SCIEX Q3200 triple quadruple mass spectrometer (AB SCIEX, USA) equipped with an electrospray ionization source (ESI) was used for the detection of the analytes in multiple reaction monitoring (MRM) modes. The operating parameters in MRM were as set as follows: the ion spray voltage (IS) of 5500 V (positive mode) and − 4500 V (negative mode); turbo spray temperature (TEM) of 500 °C; ion source gas 1 (GS 1) of 50 psi; ion source gas 2 (GS 2) of 50 psi. The declustering potential (DP) and collision energy (CE) were set to match the MRM of each analyte. Table 1 showed the summary of MS/MS detection parameters.

2.6. Method validation

2.6.1. Standard curve, limits of detection (LOD) and quantification (LOQ)

The standard solution was diluted to establish a calibration curve with a serial concentration (100%, 85%, 60%, 50%, 35%, 20% and 4%). The calibration curve was constructed based on the peak area relative to the corresponding concentration of the reference standard. The S/N ratio was calculated of the peak height divided
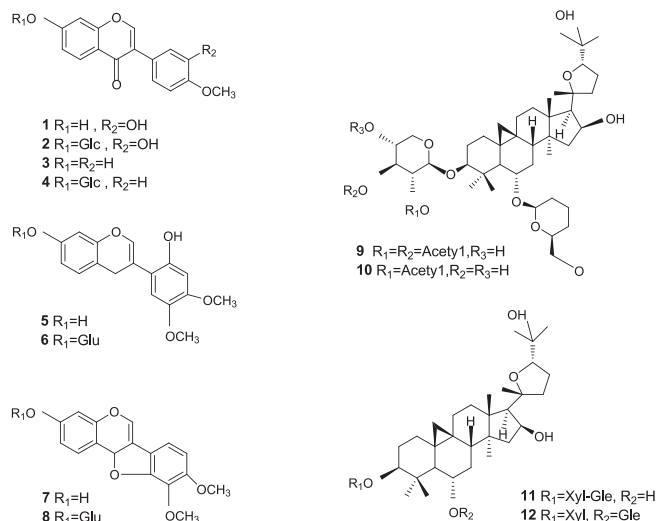

Fig. 1. Chemical structures of compounds analyzed in AR.

Table 1
Retention time and related MS information of 12 analytes detected by MRM analysis.

No.	Analytes	RT (min)	Ion mode	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (V)
1	Calycosin	13.38	ESI+	285.2	213.1	70	45
2	Calycosin-7-O-β-$_D$-glycoside	7.69	ESI+	447.3	285.4	60	20
3	Formononetin	19.69	ESI+	269.0	167.2	70	47
4	Onion	11.04	ESI+	431.1	269.1	65	20
5	(6aR, 11aR)9,10-dimethoxyptercarpan	20.11	ESI+	301.2	167.2	54	20
6	(6aR, 11aR)9,10-dimethoxyptercarpan-3-O-β-$_D$-glucoside	12.44	ESI+	463.3	167.4	60	40
7	7,2′-Dihydroxy-3′,4′-dimethoxy isoflavan	20.45	ESI+	303.0	167.2	55	19
8	7,2′-Dihydroxy-3′,4′-dimethoxyisoflavane-7-O-β-$_D$-glucoside	16.53	ESI−	463.3	301.0	−74	−24
9	Astragaloside I	21.91	ESI−	867.9	59.1	−250	−83
10	Astragaloside II	20.93	ESI−	825.7	59.1	−110	−70
11	Astragaloside III	20.42	ESI−	783.8	160.9	−150	−47
12	Astragaloside IV	20.42	ESI−	783.8	101.0	−115	−57

by the background noise. The LOD and LOQ of each analyte were determined at the S/N ratios of 3 and 10, respectively.

2.6.2. Precision, repeatability and stability

The QC sample was prepared by mixing the standard solutions to perform precision, repeatability and stability. Precision was investigated by analyzing six replicates of the QC sample in 1 d. The precisions were evaluated by calculating the variations of the peak areas, and the relative standard deviations (RSD) were chosen to represent. Repeatability was carried out by analyzing six new prepared AR samples in three separate days. In order to evaluate the stability, the sample of mixed standard solutions was analyzed at 0, 4, 8, 12, 24 and 48 h, respectively.

2.6.3. Accuracy (% recovery)

The recovery test was determined by adding low (50%), medium (100%) and high (150%) concentrations of the mixed standard solutions to AR sample, and calculating the content of each component according to the calibration curve to determine the recovery test.

2.7. Statistical analysis

All statistical analyses were performed using One-way ANOVA by SPSS 16.0 (version 16.0, Chicago, IL, USA), and the value at $P < 0.05$ was considered as significance threshold.

3. Results and discussion

3.1. Optimization of analytical method

In the early stage of the experiment, we tried to find a suitable LC-MS method for simultaneous determination of all 12 compounds in AR samples. Different chromatographic columns, mobile phase composition and ratio, and detection ion mode were firstly optimized. Here, we compared the separations between two chromatographic columns, namely, ethylene bridged hybrid (BEH) C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters) and high strength silica (HSS) T3 column. The BEH C_{18} column was a general-purpose column choice for UHPLC separation, while HSS T3 column was used to retain and separate polar organic compounds that are smaller and more water-soluble than BEH C_{18} column. The separation efficiencies of the 12 analytes were compared between these two chromatographic columns. The results showed that the HSS T3 column had better retention and chromatographic separation effects for 12 analytes. Different mobile phases were also investigated including acetonitrile-acid aqueous solution, acetonitrile–water and methanol–water, as well as acetic acid, formic acid and other solvents with good chromatographic behavior. Finally,

we found that acetonitrile-0.2% formic acid aqueous solution (volume percentage) had the best separation effect on the analyzed compounds.

To establish a fast and feasible quantitative method, parent ions and daughter ions of 12 analytes were optimized according to their mass spectra. It was found that compounds (**1–7**) had higher intensity in the positive ion mode, while compounds (**8–12**) have better strength in negative ion mode. Therefore, these analytes were measured in two modes in the MRM analysis (Table 1). However, it was found that these components could not be determined simultaneously by one method. The well separations of compounds **1–7** and **8–12** were observed in positive and negative ions, respectively. Thus, we decided to carry out two batches of separation analysis under different ionic modes. Fig. 2 showed the typical MRM chromatograms of 12 analytes in positive (A) and negative (B) modes, respectively.

3.2. Analytical method validation

3.2.1. Linearity, limits of detection and quantification

The mixed standard stock solution was diluted to an appropriate concentration with 100%, 85%, 60%, 50%, 35%, 20%, 4% to establish a calibration curve. As showed in Table 2, all the calibration curves exhibited good linearity (R^2 > 0.9983) within the test ranges. The LOD and LOQ were measured as SN > 3 and SN > 10, respectively. And the LODs and LOQs of 12 analytes were in the range of 0.0249–4.2800 ng/mL and 0.0311–8.5600 ng/mL, respectively.

3.2.2. Precision, repeatability, and stability

The results showed that all the precision, repeatability and stability of the 12 analytes were less than 4.92 %, indicating that the method had good precision, repeatability and stability (Table 3).

3.2.3. Accuracy

The recovery test was evaluated by measuring the percentage recovery of 12 analytes. The results showed that the overall recoveries ranged from 95.13% and 106.52% with RSDs less than 4.90% (Table 4).

3.3. Experimental sample determination

The mass spectrometry analysis of the samples was shown in Fig. 2, and the content of each chemical was calculated using the standard regression equation of each compound (Table 5). In the wild-simulated AR samples, the contents of calycosin, calycosin-7-O-β-$_D$-glycoside, formononetin, onion, (6aR, 11aR) 9,10-dimethoxyptercarpan, (6aR, 11aR) 9,10-dimethoxyptercarpan−3-
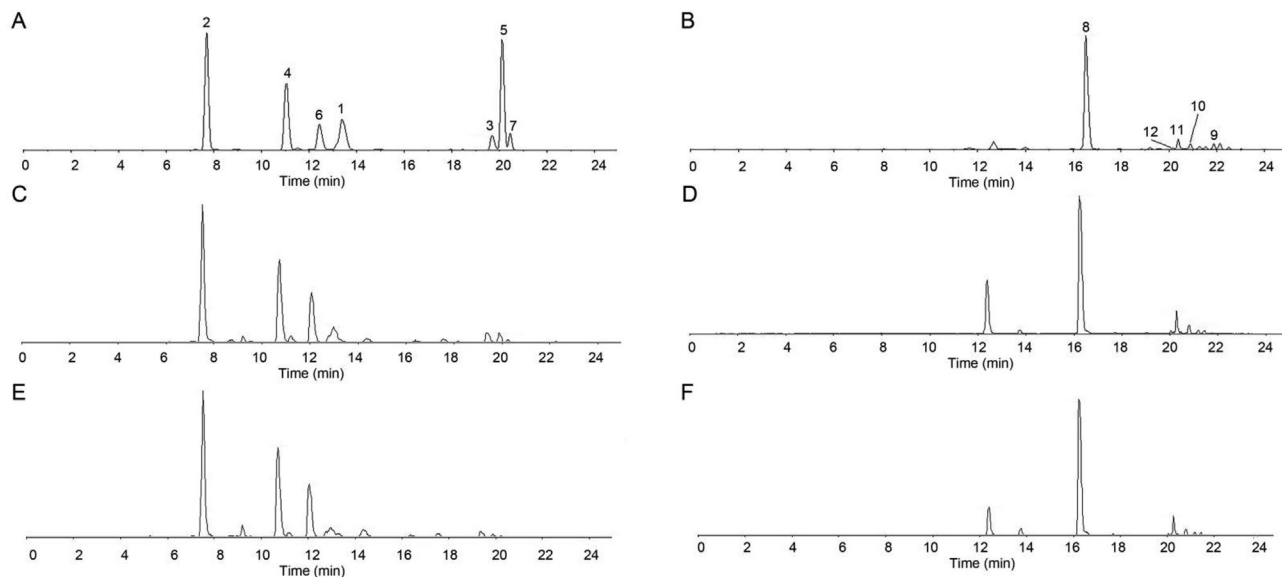
Fig. 2. MRM chromatograms of 12 analytes in positive (A, C, E) and negative (B, D, F) modes, respectively. Mass spectrometry detection of wild-simulated (C, D) and cultivated AR (E, F). (A, B: reference substances).

Table 2
Linearity, LODs and LOQs of 12 analytes detected by MRM analysis.

No.	Calibration curves	R^2	Range (µg/mL)	LOD (ng/mL)	LOQ (ng/mL)
1	$y = 202558x + 365088$	0.9992	0.864–21.600	0.1080	0.0311
2	$y = 315998x + 1000000$	0.9990	1.523–38.064	0.0381	0.0761
3	$y = 446765x + 322603$	0.9989	0.475–11.880	0.0594	0.1663
4	$y = 569026x + 226845$	0.9985	0.698–17.440	0.0249	0.0872
5	$y = 469388x + 141426$	0.9991	0.696–17.410	0.3484	0.8705
6	$y = 94508x + 1901.1$	0.9993	0.257–6.427	0.1328	0.3994
7	$y = 51545x + 4316.5$	0.9993	0.620–15.500	0.5061	1.6240
8	$y = 272274x + 1000000$	0.9983	1.872–46.800	0.0016	0.0062
9	$y = 73.424x + 2036.5$	0.9994	8.560–214.00	4.2800	8.5600
10	$y = 9645.8x + 22021$	0.9995	2.448–61.200	0.8136	2.5628
11	$y = 4920.4x + 9171.7$	0.9989	1.128–28.200	0.0141	0.0493
12	$y = 5356.3x + 33077$	0.9990	3.820–95.500	1.8520	5.7364

Table 3
Precisions, repeatability and stability of 12 analytes detected by MRM analysis ($n = 6$).

Analytes	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)
1	1.31	3.43	2.95
2	2.03	4.49	3.73
3	2.06	4.47	3.93
4	1.46	4.31	3.00
5	1.65	4.09	3.37
6	1.96	4.92	3.77
7	1.42	4.63	4.05
8	1.25	3.68	1.50
9	2.36	4.84	2.52
10	1.65	2.66	2.03
11	1.41	2.65	1.87
12	2.18	4.67	2.06

O-β-D-glucoside, 7,2′-dihydroxy-3′,4′-dimethoxyisoflavan, and 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-O-β-D-glucoside were higher than those of cultivated AR, while the contents of astragaloside III, astragaloside IV in cultivated AR were higher than those of wild-simulated AR. In addition, we also compared the content of total flavonoids and saponins in wild-simulated and cultivated AR. The results showed that the total flavonoid content in wild-simulated AR was higher than cultivated AR, while the content of saponins was higher in cultivated AR than wild-simulated AR (Fig. 3). The literature survey found that the content of flavonoids in the cultivated AR was lower than that of the wild-simulated (Wan, 2015), which were same as our results. For the saponins components, the literature (Song, Zhou, Zhu, & Zhao, 2020) showed that the content of astragaloside III in the cultivated AR was higher than wild-simulated, and the content of astragaloside IV in the cultivated samples was lower than the wild-simulated samples. But the results of our study showed that the content of astragaloside IV was higher than wild-simulated, the reason might be that the cultivated AR used in this experiment is a fourth-class product, and the content of astragaloside IV was the highest. Based on this result, it could provide reference for choosing different kinds of AR for clinical treatment of diseases.

In recent years, the research on the proportional relationship of the chemical components of TCM has been widely used in the quality evaluation of TCM and the quality research of authentic medicinal materials. Wei (Wei et al., 2004) used HPLC to determine the content of ligustilide and senkyunolide in *Ligusticum chuanxiong* produced in the local area in six years. The content of ligustilide and senkyunolide was 1.5%–2.2% and the ratio of ligustilide and senkyunolide is basically fixed between 2.3 and 2.6, which showed that the uniqueness of the internal chemical composition of authentic medicinal materials. In our study, the flavonoids content in wild-simulated AR was higher than the cultivated AR, while the saponins in cultivated AR was higher than the wild-simulated AR. The further comparison showed the ratio of saponins/flavonoids in

Table 4
Recovery results of 12 analytes detected by MRM analysis.

Analytes	Observed amount (μg)	Origin amount (μg)	Spiked (μg)	Recovery (%)	RSD (%)
1	5.865	2.932	8.905	103.70	3.36
		5.865	11.605	97.88	1.64
		8.797	14.493	98.09	3.02
2	7.559	3.779	11.508	104.49	3.76
		7.559	15.470	104.67	4.51
		11.338	18.895	99.99	3.22
3	0.806	0.403	1.189	95.13	1.49
		0.806	1.632	102.63	1.19
		1.208	2.009	99.60	2.16
4	1.986	0.993	3.015	103.61	3.70
		1.986	4.042	103.56	2.22
		2.979	4.848	96.11	4.24
5	1.534	0.767	2.279	97.13	4.76
		1.534	3.064	99.74	3.91
		2.300	3.942	104.70	4.80
6	1.979	0.990	3.015	104.69	2.68
		1.979	3.974	100.78	2.83
		2.969	5.142	106.52	2.70
7	0.584	0.292	0.863	95.76	1.20
		0.584	1.191	104.14	2.14
		0.875	1.432	96.96	1.37
8	4.558	2.279	6.794	98.13	3.62
		4.558	9.114	99.98	2.54
		6.836	11.643	103.65	4.38
9	47.141	23.571	94.220	103.25	3.42
		47.141	71.478	99.87	4.07
		70.712	119.449	102.26	4.77
10	23.778	11.889	35.568	99.18	2.69
		23.778	46.977	97.57	1.99
		35.666	59.588	100.40	2.09
11	15.636	7.818	23.689	103.00	4.62
		15.636	31.249	99.85	1.24
		23.455	39.170	100.34	4.90
12	36.955	18.478	55.574	100.77	3.08
		36.955	74.105	100.53	4.82
		55.433	92.603	100.39	3.93

Table 5
Determination results of compounds in wild-simulated and cultivated AR (W: wild-simulated AR; C: cultivated AR).

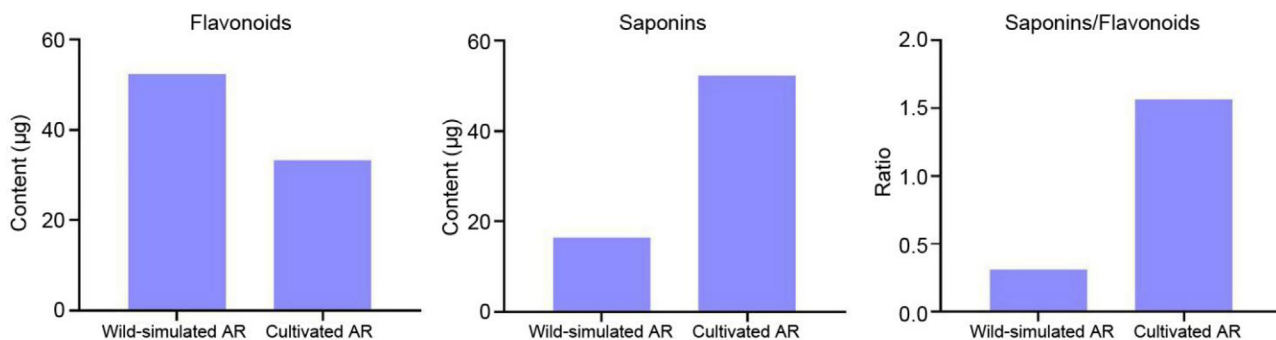| Analytes	Wild-simulated AR			Cultivated AR		
	W1	W2	W3	C1	C2	C3
1	20.94949968	20.91150577	20.5064589	14.05072184	14.24310407	13.99122336
2	9.50301642	9.700490724	9.281430504	4.467421677	4.620578797	4.91173886
3	1.449077255	1.339399908	1.129045214	0.653685942	0.642560406	0.632092935
4	5.580461764	5.244714783	4.938922028	4.416538896	4.246968764	4.707519613
5	2.383047713	2.127395673	2.356960979	0.948874705	0.933961669	0.978700776
6	9.026737419	8.540006137	8.233153807	4.942427096	4.68848034	4.953008211
7	5.096197497	4.358977592	4.378378116	3.641158211	3.699359783	3.583519255
Analytes	Wild-simulated AR			Cultivated AR		
	W4	W5	W6	C4	C5	C6
8	4.260414142	4.370597266	4.203320185	2.183296238	2.056751655	2.35057332
11	2.099077311	2.131635639	2.306458824	8.460348752	8.521319405	8.433554183
12	10.02987137	9.920144316	9.873087206	41.63170472	40.8720572	42.17892948

Fig. 3. Content of flavonoids and saponins in wild-simulated and cultivated AR and ratio of saponins/flavonoids in both AR.

cultivated AR was higher than that of wild-simulated AR (Fig. 3), which might be the real uniqueness difference of them. The efficacy of AR is usually the joint action of all the components, and further studies on other components and their ratios are still needed.

4. Conclusion

In this research, an accurate, fast, efficient, and practical UPLC-MS method was developed for the quantitative analysis of 12 chemical components in wild-simulated AR and cultivated AR. The results showed that the components of flavonoids in wild-simulated AR are higher than those of the cultivated AR, the saponins in cultivated AR are higher, and the ratio of saponins/flavonoids in cultivated AR was higher. This article analyzed several chemical components of AR under different planting methods and found some differences between the two planting methods. However, a comprehensive analysis of the chemical components of AR has not yet been conducted, and more differences in pharmacology need to be further explored.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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