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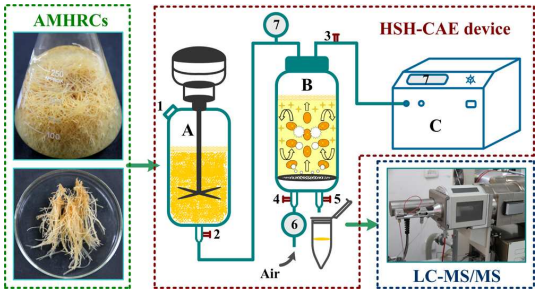


HSH-CAE-LC-MS/MS opened up a new avenue for the direct determination of secondary metabolic profiles from fresh plant *in vitro* cultures.

# Direct determination of astragalosides and isoflavonoids from fresh *Astragalus membranaceus* hairy root cultures by high speed homogenization coupled with cavitation-accelerated extraction followed by liquid chromatography-tandem mass spectrometry

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**Abstract**

A direct analysis approach for plant *in vitro* cultures, namely high speed homogenization coupled with cavitation-accelerated extraction (HSH-CAE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), was developed for simultaneous determination of six astragalosides and five isoflavonoids in *Astragalus membranaceus* hairy root cultures (AMHRCs). In comparison to reported soxhlet extraction (SE) and ultrasound-assisted extraction (UAE) methods, the proposed sample preparation procedure (HSH-CAE) offers significant improvements with regard to simplicity in operation (elimination of biomass drying and grinding), high efficiency, enhanced yield and green aspects in terms of saving energy cost and minimizing the generation of waste. Additionally, the HSH-CAE mechanism was clarified via cytohistological studies of samples at cellular/tissular levels. Moreover, the established LC-MS/MS method provided linearity with correlation coefficients above 0.9991, limit of detections (LODs) below 1.77 ng $mL^{-1}$, relative standard deviations (RSDs) below 6.01%, and recoveries above 96.84%. Furthermore, the proposed HSH-CAE-LC-MS/MS method was also successfully applied for screening high-productive AMHRCs. Overall, this study opened up a new avenue for the direct determination of secondary metabolic profiles from fresh plant *in vitro* cultures, which was valuable for improving the quality control of plant cell/organ cultures and shed light on the metabolomics analysis from biological samples.

## 1. Introduction

*Astragalus membranaceus* (Fisch.) Bunge roots (Radix astragali) are commonly used in United States, Europe and Asian countries as traditional medicines for the treatment of cardiovascular disease, cancers, diabetes mellitus, nephritis, leukemia, hypertension and hyperhidrosis, or as health-promoting foodstuffs (typically soups and teas) to enhance the human immune system and to reinforce the body vital energy [1–4]. However, the limited supply (endemic plant species for East Asia) together with the over-exploitation constitutes the most important hurdles for developing Radix astragali-based drugs or dietary supplements [5, 6]. To address these issues, it has become feasible to use plant *in vitro* cultures for the large-scale production of valuable phytochemicals from this species [4]. In this context, *A. membranaceus* hairy roots cultures (AMHRCs) generated from the genetic transformation of *Agrobacterium rhizogenes*, have emerged as attractive alternatives to produce active compounds effectively, economically and in an environmentally friendly way [6–9].

Astragalosides and isoflavonoids, the principal active ingredients of Radix astragali, possess versatile biological activities as diverse as anti-tumor, cardiotonic, immunomodulatory, neuroprotective, antiviral, antioxidant, anti-inflammatory, antiperspirant, hepatoprotective, antihypertensive, anti-fatigue, anti-osteoarthritis effects, etc. [4, 10–12]. Accordingly, it is necessary to establish a valid analytical method to characterize the major pharmaceuticals (astragalosides and isoflavonoids) and efficiently control the quality of AMHRCs. It is known the fact that the analysis of metabolites in plant *in vitro* cultures is a challenging task because of their chemical diversity, usually low abundance and variability within different cell/organ lines [13]. Liquid chromatography-mass spectrometry (LC-MS/MS) offers excellent sensitivity and selectivity, combined with the ability to elucidate or confirm chemical structures

of target constituents in complex biological samples based on their exact MS/MS fragment patterns [14, 15]. Sample preparation is still the most tedious and time-consuming step, which is recognized as the main bottleneck of the analytical process [16].

Extraction of secondary metabolites from plant cell/organ cultures is always limited by their high water contents. Conventional sample preparation methods such as soxhlet extraction (SE) and ultrasound-assisted extraction (UAE), are available for phytochemicals extraction from plant *in vitro* cultures, but they generally require high energy consumption for dewatering and drying pretreatment along with long duration and low efficiency [17, 18]. Therefore, development of new sample preparation strategies for HRCs that eliminate biomass drying and enhance extraction efficiency can lead to significant energy and cost savings. Moreover, selection of a particular sample preparation method should depend on the simplicity of the extraction technique and its convenience. High speed homogenization (HSH) has been an effective sample pretreatment technique, which can facilitate the destruction of fresh plant materials for a better access to intracellular substances [19, 20]. In addition, cavitation-accelerated extraction (CAE) developed by our laboratory, is a simple, environmentally friendly and efficient technology, which has been successfully applied in the extraction and analysis of bioactive ingredients from several medicinal plants [21, 22]. However, CAE has never been used for the extraction of phytochemicals from plant *in vitro* cultures.

In this work, HSH coupled with CAE (HSH-CAE) followed by LC-MS/MS was proposed for the direct determination of six astragalosides and five isoflavonoids in AMHRCs. The diagram of work flow is shown in Fig. 1. Various influential parameters of the proposed sample preparation method were optimized systematically. Subsequently, the superiority of HSH-CAE was evaluated as compared to

conventional methods in terms of extraction efficiency and green aspects. Moreover, cytohistological studies of samples before and after extraction were performed to clarify the extraction mechanism. Furthermore, a sensitive and accurate LC-MS/MS method with selected reaction monitoring (SRM) model for simultaneous quali-quantitative analysis of eleven target compounds was successfully established. The method validation of the proposed approach was also investigated. Eventually, the developed analytical method was performed for screening high-productive AMHRCs among eight candidates.

## 2. Materials and methods

### 2.1. Materials and reagents

Eight *A. membranaceus* hairy root lines (I–VIII) were successfully induced via the genetic transformation of *A. rhizogenes* LBA9402 in our laboratory. Eight AMHRCs (I–VIII) were initiated by culturing 1.5 g (fresh weight, FW) of different hairy root lines in 250 mL Erlenmeyer's flasks containing 150 mL of Murashige and Skoog (MS)-based liquid medium (pH 5.8) supplemented with 30 g $L^{-1}$ sucrose and 1 g $L^{-1}$ casein hydrolyzate but without $NH_4NO_3$, and incubated on a rotary shaker (100 rpm) in the dark at 25 ± 1 °C. AMHRCs were harvested by filtration after 4 weeks of cultivation, and then rinsed with tap and distilled water. Moisture contents of eight AMHRCs (I–VIII) were pre-determined for the further quantitative analysis.

Astragalosides and isoflavonoids standards including including astragaloside I (AG I), astragaloside II (AG II), isoastragaloside II (IAG II), astragaloside III (AG III), astragaloside IV (AG IV), cycloastragenol (CY), calycosin-7-O-β-D-glucoside (CAG), ononin (ON), astraisoflavan-7-O-β-D-glucoside (ASG), calycosin (CA) and formononetin (FO) were purchased from Weikeqi Biological Technology Co. Ltd.

(Sichuan province, China). Other reagents of either analytical or optical grade were obtained from Beijing Chemical Reagents Co. (Beijing, China).

## 2.2. HSH-CAE procedure

### 2.2.1. Extraction process

The HSH-CAE instrument was designed and manufactured by our laboratory. As the diagram illustrated in Fig. 1, the apparatus consists mainly of a high speed homogenizer (A), a cavitation chamber (B) and a vacuum pump (C). During the extraction process, the fresh AMHRCs VI (5.0 g, FW) were initially added into the homogenizer from an inlet (1) while all the valves were kept closed. After HSH treatment, valves (2) and (3) and the vacuum pump were turned on successively. Meanwhile, the homogenates and extraction solvents were introduced automatically into the cavitation chamber by the generated negative pressure. Subsequently, the valve (2) was turned off but the valve (4) was turned on, and then the continuous air flow was introduced into the cavitation chamber for CAE process. The vacuum degree of the extraction system was monitored by the throttling gauge (6) and the pressure gauge (7). Additionally, a sieve plate set in the bottom of the cavitation chamber was utilized to generate cavitations with different intensities and characteristics. After the extraction, valves (3) and (4) and the vacuum pump were turned off successively. The vacuum in the cavitation chamber was released by adjusting the valve (2). Eventually, the extraction solvent was filtered through the sieve plate under gravity, and collected via the valve (5). The obtained solution was then centrifuged and filtered through a 0.22 μm nylon membrane for LC-MS/MS analysis.

### 2.2.2. Experimental design

To achieve the optimum efficiency by HSH-CAE, Box-Behnken design (BBD) [23] was applied to survey the effect of four key independent variables at three levels

(negative pressure -0.06 to -0.09 MPa, homogenization time 30 to 60 s, liquid/solid ratio 3 to 8, and extraction time 10 to 30 min) on the dependent variable (the sum yields of AG I, AG II, IAG II, AG III, AG IV, CY, CAG, ON, ASG, CA and FO). Liquid/solid ratio was calculated based on the fresh weight of hairy roots. The actual and coded levels of the independent variables used in the experimental design are summarized in Table S1. The experiment data were analyzed statistically with Design-Expert 7.0 software (State-Ease, Inc., Minneapolis MN). Analyses of variance (ANOVA) were performed to calculate and simulate the optimal values of the tested parameters.

**2.3. Conventional procedures**

The washed AMHRCs VI were dried in a vacuum drier at 60 ℃ till a constant weight, and then the dry materials were ground to fine powders and extracted by the reported soxhlet extraction (SE) and ultrasound-assisted extraction (UAE) methods with slight modifications [17, 18]. For SE: root powders (0.5 g) were placed in a Soxhlet apparatus and extracted with 80% ethanol solution (25 mL) at 90 ℃ for 4 h. For UAE: root powders (0.5 g) were extracted with 80% ethanol solution (25 mL) in an ultrasonic bath (KQ-250DB, Kun-shan Ultrasonic Instrument Co. Ltd., China) for 120 min. After the extraction of each method, the obtained solutions were centrifuged and filtered through a 0.22 μm nylon membrane for LC-MS/MS analysis.

**2.4. LC-MS/MS analysis**

An Agilent 1100 series HPLC (Agilent, San Jose, USA) coupled to an API 3000 triple tandem quadrupole MS (Applied Biosystems, Concord, Canada) equipped with a Phenomenex Gemini C18 110A reversed-phase column (250 mm × 4.6 mm I.D., 5 μm) was applied for the analysis of target compounds from AMHRCs. The binary mobile phase consisted of acetonitrile (A) and 0.01% formic acid aqueous solution (B)

using the gradient program as follows: 0–3 min, 45% (A); 3–8 min, 45–50% (A); 8–13 min, 50–60% (A); 13–18 min, 60–65% (A); and 18–20 min, 65–45% (A). The column temperature was maintained at 30 °C, the flow rate 1.0 mL $min^{-1}$ and the injection volume 10 μL.

All mass spectra of target analyses were acquired in SRM mode with electrospray ionization (ESI) source operating in the negative ion mode. The universal parameters were as follows: nebulising gas, curtain gas and collision gas set as 12, 10 and 6 a.u. (arbitrary units), respectively; ion source temperature 300 °C; ion spray voltage -4500 V; focusing potential and entrance potential set as -75 and -10 V, respectively. To obtain the highest response for each analyte, the specific parameters for acquiring the optimal precursor/product ion combinations including declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized and summarized in Table 1. Contents of target compounds were calculated by corresponding calibration curves based on the dry weight of roots. For fresh AMHRCs, the dry weights were obtained through converting fresh weights by the aid of their moisture contents.

### 2.5. Statistical analysis

All experiments in this work were conducted for three times. Results were expressed as means ± standard deviations. The data were statistically analyzed using the SPSS statistical software, version 17.0 (SPSS Inc, Chicago, Illinois, USA). Differences between means were determined by analysis of variance (ANOVA) with Duncan's test on the level of significance declared at $P < 0.05$.

## 3. Results and discussion

### 3.1. Optimization of HSH-CAE conditions

The rationale for application of the HSH pretreatment is various, the most

evident being the ability of a high speed homogenizer (12000 rpm) to handle fresh plant materials in a continuous stream with turbulence, shear stress and friction, which can affect morphological changes in plant matrix that enhances the following extraction process [19, 20]. Additionally, the cavitation of CAE in this work is generated by negative pressure via vacuum pump, and air is introduced continuously into the extraction vessel along with the collapse of bubbles that creates intensive cavitation-collision, turbulence, suspension and interface effects for disrupting plant cells and accelerating mass transfer [21, 22].

According to results of our preliminary experiments (data not shown), 80% ethanol solution was adopted for the simultaneous extraction of six astragalosides and five isoflavonoids. Considering that the numbers of experiments necessary for optimizing extraction conditions can be reduced by statistical experimental design, the homogenization time, negative pressure, liquid/solid ratio and extraction time on the yield of total target analytes were optimized by BBD combined with response surface methodology (RSM) [24]. The experimental design matrix and all the related data are illustrated in Table S1.

### 3.1.1 Fitting the mathematical model

ANOVA results of the built quadratic model are presented in Table S2. A highly significant level of the model ($P < 0.0001$), a not significant "lack of fit" ($P > 0.05$) and a desirable determination coefficient ($R^2 = 0.9843$) suggested that the built mathematical model was precise and applicable. Moreover, the factors with the significant effects ($P < 0.05$) on the dependent variable of the model were the linear terms of $X_1$, $X_2$, $X_3$ and $X_4$, interaction term of $X_1X_4$, $X_2X_4$ and $X_3X_4$, and quadratic terms of $X_1^2$, $X_2^2$, $X_3^2$ and $X_4^2$. However, the interaction terms of $X_1X_3$, $X_1X_3$ and $X_2X_3$ were insignificant ($P > 0.05$) and could be negligible. The second-order polynomial

model was applied to express the extraction efficiency of total target analytes as the following equation:

$$Y = 2.79 - 1.11X_1 + 0.13X_2 - 0.092X_3 + 0.043X_4 + 0.065X_1X_4 - 0.08X_2X_4 - 0.11X_3X_4 - 0.52X_1^2 - 0.095X_2^2 - 0.3X_3^2 - 0.067X_4^2$$

Where $Y$ was the yield of total target analytes; $X_1$ was the negative pressure (MPa); $X_2$ was the homogenization time (s); $X_3$ was liquid/solid ratio (mL $g^{-1}$); and $X_4$ was the extraction time (min).

**3.1.2. Analysis of the response surface**

As shown in Fig. 2A, both negative pressure and liquid/solid ratio exhibited significantly double effect on the yield of target analytes, and it demonstrated that negative pressure around -0.08 MPa and liquid/solid ratio around 5 resulted in high recovery. As presented in Fig. 2B, the yield of target analytes increased with the decrease of negative pressure from -0.06 to -0.08 MPa at a fixed homogenization time, but decreased significantly afterwards. This phenomenon is closely related to the cavitation effect of CAE that can promote the extraction of intracellular substances by means of disrupting plant cells and accelerating mass transfer [21, 22]. Factually, the cavitation effect increases initially with the decrease of negative pressure, but diminishes expeditiously with the further decrease of negative pressure due to the lack of air to form cavitation bubbles [21]. As exhibited in Fig. 2C, with increasing liquid/solid ratio from 3 to 5 at a given extraction time, the yield of target analytes increased accordingly, however, the further increase in liquid/solid ratio resulted in a significant decrease in the recovery. The inadequate solvent can promote mass transfer barrier as the distribution of intracellular compounds is concentrated in certain regions which limits the movement of them out of cell matrix. However, the excessive solvent can consume the cavitation energy of CAE thus resulting in the poor extraction

efficiency. It was observed from Fig. 2D that the yield of target analytes increased with the extension of homogenization time from 30 to 54 s at a fixed liquid/solid ratio but increased slightly afterwards. The extended homogenization time did not improve extraction yield significantly as the target ingredients may have leached out from sample matrices before extraction. As seen from Fig. 3E and F, at a given negative pressure or homogenization time, the yield of target analytes increased with extended time initially but stabilized beyond 22 min, which was likely ascribable to the exhaustion of target compounds in AMHRCs.

### 3.1.3. Verification of the predictive model

Based on the mathematical model built, the optimal experimental parameters were as follows: negative pressure -0.077 MPa, homogenization time 55.7 s, liquid/solid ratio 5.21 and extraction time 19.28 min. Considering the actual operation, the homogenization time, liquid/solid ratio and extraction time were modified slightly to 56 s, 5.2 and 19.3 min, respectively. To validate the reliability of the theoretical model prediction, three sequential experiments were performed under optimal parameters. The yield of total target analytes was 2.81 ± 0.09 mg $g^{-1}$ from the actual experiments, which was a good fit for the value (2.85 mg $g^{-1}$) forecasted by the regression model. Therefore, the optimal extraction conditions obtained were reliable and practical.

## 3.2. HSH-CAE predominance

In the previous reports, conventional SE and UAE methods were utilized for the extraction of phytochemicals from dried plant hairy root cultures [17, 18]. Therefore, the superiority of the proposed HSH-CAE approach was evaluated against these traditional methods in this work. The obvious advantages of HSH-CAE were mainly reflected as follows: simplicity in operation (elimination of biomass drying and

grinding), highest efficiency (20.2 min as against 240 min of SE and 120 min of UAE), improvable yield (2.81 mg $g^{-1}$ as against 2.69 mg $g^{-1}$ of SE and 2.57 mg $g^{-1}$ of SE), lowest energy cost (0.021 kWh $mg^{-1}$ as against 15.94 kWh $mg^{-1}$ of SE and 15.32 kWh $mg^{-1}$ of UAE) and minimal $CO_2$ generation (0.017 Kg $mg^{-1}$ as against 12.75 Kg $mg^{-1}$ of SE and 12.26 Kg $mg^{-1}$ for UAE). The energy consumption was determined with a Wattmeter based on the extraction of 1 mg total target analytes. The calculation of $CO_2$ ejected was made according to the previous literature [25].

Conventional mechanical grinding technology for dried biomasses always causes the local overheating of materials thus leading to the thermal degradation of some susceptible compounds. However, HSH technique can effectively pulverize fresh plant materials in a continuous slurry stream and avoid the localized increased temperatures. In addition, traditional extraction methods always need long-term heating at high temperature, which will result in the degradation of thermolabile compounds. Conversely, CAE was performed with air flow at room temperature, thus, the thermal degradation of sensitive analytes could be reduced or prevented. These beneficial properties of HSH-CAE contributed to an increase in extraction yields of target compounds. Consequently, the proposed HSH-CAE is a simple, low cost, green and effective method for the direct and augmentation extraction of astragalosides and isoflavonoids from fresh AMHRCs.

### 3.3. HSH-CAE mechanism

Since cell walls and membranes present formidable barriers to permeation by extraction solvents, cells have to be disrupted prior to extraction. Herein, the cytohistological analysis of AMHRCs samples before and after each procedure was performed to clarify the HSH-CAE mechanism. The micrograph of untreated AMHRCs is shown in Fig. 3A. After HSH (Fig. 3B), AMHRCs were obviously

dispersed from an intact organ into numerous cells (arrows). After CAE (Fig. 3C), AMHRCs cells exhibited an evident rupture of cellular matrix (arrows), and a movement of intracellular substances into the solvent media could be thus envisaged. During extraction process, the intensive cavitation-collision effect along with the collapse of bubbles can penetrate the surface of cellular matrix, thus resulting in the effective disintegration of plant cells. The conclusive phenomenon in this study suggested that HSH-CAE was an efficient hyphenated sample preparation technique for the extraction of intracellular phytochemicals from plant *in vitro* cultures.

### 3.4. Establishment of LC-MS/MS method

#### 3.4.1. Optimization of LC conditions

The fundamental basis of an efficient chromatographic process is the development of a suitable mobile phase with an appropriate elution program, which can achieve the best possible resolution and ionization of analytes in LC-MS/MS analysis. According to our previous report, the acetonitrile-water mobile phase is the best choice of separation and ionization of astragalosides and isoflavonoids [8, 9]. Therefore, the mobile phase composition was simplified by using an acetonitrile-water mixture for all analytes in this work. Additionally, the presence of acid could improve the chromatographic behavior and reduce the peak tailing in the present study. On the other hand, the addition of acid was not beneficial for the deprotonation of analytes in the following ESI-MS/MS process with negative ion mode. Taking into account the balance between the above two aspects, a solvent system consisting of acetonitrile and 0.01% formic acid aqueous solution, which could provide the satisfactory baseline stability and ionization efficiency, was ultimately selected as mobile phase. Moreover, the developed gradient elution program as described in Section 2.4 offered a short run time (20 min) and sufficient

resolution of eleven target analytes with very little matrix effects in the following ESI-MS/MS analysis.

### 3.4.2. Optimization of ESI-MS/MS parameters

In our previous study, the ESI-MS/MS measurements of astragalosides in positive ionization mode could provide higher response sensitivity as against those in negative ionization mode [8]. However, the signals of isoflavonoids were hardly detected in positive ionization mode but could be easily caught in negative ionization mode [9]. For the simultaneous determination of all target analytes, the ESI-MS/MS with negative-ion mode was eventually chosen to analyze the eleven target compounds in spite of sacrificing the detection sensitivity of astragalosides. To obtain the most informative fragmentation spectrum in ESI-MS/MS with SRM model, several critical parameters including DP, CE and CXP on the signal intensities of all target analytes were investigated systematically. Results of these parameters were optimized manually, acquired and summarized in Table 1.

Under the optimal conditions, the product ions mass spectra of CAG, ON, ASG, AG IV, AG III, CA, AG II, IAG II, AG I, FO and CY are presented in Fig. 4B–L, respectively. The ESI-MS/MS analysis of CAG produced a precursor ion of *m/z* 445.4 ($[M-H]^-$), which produced a fragmentation pattern dominated by an ion at *m/z* 283.0 ($[(M-H\text{-glucose}]^-$) (Fig. 4B). Therefore, the mass transition pattern *m/z* 445.4→283.0 with the highest intensity was chosen for the identification and quantification of CAG. Likewise, the SRM transitions at *m/z* 371.0→356.1, *m/z* 429.1→266.9, *m/z* 463.3→301.1, *m/z* 783.6→160.9, *m/z* 283.1→268.0, *m/z* 825.6→765.6, *m/z* 868.7→807.4, *m/z* 267.1→252.0, *m/z* 489.6→383.3 and *m/z* 463.3→301.1 were selected for monitoring ON, ASG, AG IV & AG III, CA, AG II & IAG II, AG I, FO and CY, respectively. The representative total ion chromatogram with SRM model of

standard mixture is shown in Fig. 4A. Obviously, the established LC-MS/MS method achieved a rapid separation of all target compounds without sacrificing resolution.

### 3.5. Method validation

As the results summarized in Table 2, all calibration curves exhibited an excellent linearity ($R^2 \geq 0.9991$) within the range of tested concentrations. Limit of detections (LODs) for all target analytes were less than 1.77 ng $mL^{-1}$. Relative standard deviations (RSDs) of intra- and inter-day measurements for the retention time of all target analytes were less than 0.58% and 0.84%, respectively, and for the peak area were less than 3.88% and 6.01%, respectively. Recoveries of standard additions of all target analytes were ranging from 96.84% to 104.76%. Overall, the aforementioned data indicated that the present method possessed good accuracy and sensitivity for the quantification of target astragalosides and isoflavonoids in AMHRCs.

### 3.6. Application for screening high-productive AMHRCs

Owing to the uncertainty of *A. rhizogenes* T-DNA integration into the host plant genome, different *A. membranaceus* hairy root lines derived often show considerable diverse biosynthesis patterns of secondary metabolites [26]. Therefore, the proposed HSH-CAE-LC-MS/MS method was applied for the determination of target astragalosides and isoflavonoids in eight candidate AMHRCs (I–VIII) originated from distinct hairy root lines. Quantitative results of eleven target analytes in different cultures are presented in Table 3.

Obviously, various AMHRCs showed variations in contents of AG I, AG II, IAG II, AG III, AG IV, CY, CAG, ON, ASG, CA and FO within the ranges of 914.22–1275.39, 564.61–707.61, 171.42–211.69, 151.65–198.55, 158.76–179.88, 13.94–17.69, 6.37–10.52, 4.18–6.07, 44.56–68.27, 63.97–97.26 and 51.89–65.93, μg

374 $g^{-1}$, respectively. Among them, the levels of astragalosides (AG I, AG II, IAG II, AG
375 III, AG IV and CY) in AMHRCs VI were significantly higher as compared to other
376 candidates, while AMHRCs II was categorized as the high-productive culture in terms
377 of isoflavonoids (CAG, ON, ASG, CA and FO) accumulation. The representative
378 LC-MS/MS total ion chromatogram of AMHRCs VI sample, and the corresponding
379 extracted ion chromatograms of CAG, ON, ASG, AG IV & AG III, CA, AG II & IAG
380 II, AG I, FO and CY are shown in Fig. 5A–J, respectively. This successful application
381 example indicated that the proposed analytical method is suitable for quality control
382 of AMHRCs or other plant *in vitro* cultures.

383 **4. Conclusions**

384 In the present study, a rapid, green and effective sample preparation and
385 analytical procedure for fresh plant *in vitro* cultures, i.e. HSH-CAE method followed
386 by LC-MS/MS detection, was developed and validated for the simultaneous
387 determination of six astragalosides (AG I, AG II, IAG II, AG III, AG IV and CY) and
388 five isoflavonoids (CAG, ON, ASG, CA and FO) in AMHRCs. Operational
389 conditions of HSH-CAE were optimized systematically. Compared with reported SE
390 and UAE methods, the proposed approach exhibited predominance of easy
391 manipulation, time-saving, high yield, low energy consumption and reduced waste.
392 Cytohistological investigations provided evidences of pronounced tissular/cellular
393 damages within HSH-CAE process. Moreover, the established LC-MS/MS method
394 was proved to have excellent linearity, precision, repeatability and reproducibility.
395 The validated HSH-CAE-LC-MS/MS method was also successfully applied for the
396 selection of high-productive AMHRCs. The observed beneficial effects exerted by the
397 proposed method in this work were valuable for the rapid and valid determination of
398 secondary metabolic profiles in AMHRCs or other plant *in vitro* cultures.
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**Figure Captions:**

**Fig. 1.** The work diagram of HSH-CAE-LC-MS/MS procedure.

**Fig. 2.** Response surfaces for target analytes extraction during HSH-CAE process: (A) varying negative pressure and liquid/solid ratio; (B) varying negative pressure and homogenization time; (C) varying liquid/solid ratio and extraction time; (D) varying homogenization time and liquid/solid ratio; (E) varying extraction time and negative pressure; (F) varying extraction time and homogenization time.

**Fig. 3.** Microscopic photographs of AMHRCs samples before extraction (A), after HSH (B), and after CAE (C). Microscopic photographs were acquired via a light microscope (Leica DM 4000B) equipped with a digital camera (Nikon DS-Ri1).

**Fig. 4.** LC-MS/MS total ion chromatogram with SRM model of standard mixture (A), and the product ion mass spectra of CAG (B), ON (C), ASG (D), AG IV & AG III (E), CA (F), AG II & IAG II (G), AG I (H), FO (I) and CY (J). The elution order of target compounds as follows: 1. CAG, 2. ON, 3. ASG, 4. AG IV, 5. AG III, 6. CA, 7. AG II, 8. IAG II, 9. AG I, 10. FO, 11. CY.

**Fig. 5.** LC-MS/MS total ion chromatogram with SRM model of AMHRCs sample (A), and the corresponding extracted ion chromatograms of CAG (B), ON (C), ASG (D), AG IV & AG III (E), CA (F), AG II & IAG II (G), AG I (H), FO (I) and CY (J). The elution order of target compounds as follows: 1. CAG, 2. ON, 3. ASG, 4. AG IV, 5. AG III, 6. CA, 7. AG II, 8. IAG II, 9. AG I, 10. FO, 11. CY.

**Table 1.** Optimized MS/MS parameters for the eleven target analytes [a].

| Analytes | DP (V) | CE (V) | CXP (V) | SRM (amu) |
|---|---|---|---|---|
| AG I | -80 | -34 | -5 | 867.7 → 807.4 |
| AG II & IAG II | -124 | -36 | -13 | 825.4 → 765.5 |
| AG III & AG IV | -80 | -54 | -9 | 783.6 → 106.9 |
| CY | -195 | -40 | -12 | 489.3 → 383.3 |
| CAG | -20 | -10 | -5 | 445.2 → 283.0 |
| ON | -31 | -10 | -5 | 428.8 → 266.9 |
| ASG | -58 | -23 | -5 | 463.2 → 301.1 |
| CA | -70 | -24 | -5 | 283.0 → 268.0 |
| FO | -55 | -31 | -5 | 267.0 → 252.0 |

[a] Other parameters: nebulising gas, curtain gas and collision gas set as 12, 10 and 6 a.u., respectively; ion source temperature 300 °C; ion spray voltage -4500 V; focusing potential and entrance potential set as -75 and -10 V, respectively.

**Table 2.** Calibration curves, LODs, precision and accuracy (recovery of standard addition) for the eleven target compounds as determined by the developed HSH-CAE-LC-MS/MS method.

| Analytes | Calibration curves [a] | $R^2$ | Linear range (ng mL$^{-1}$) | LOD [b] (ng mL$^{-1}$) | Intra-day RSD (%) [c] | | Inter-day RSD (%) | | Standard addition recovery (%) [f] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Rt [d] | PA [e] | Rt | PA | Mean ± RSD (n=3) |
| AG I | $Y = 986\,X + 2570$ | 0.9991 | 8.64–8640 | 0.48 | 0.27 | 1.91 | 0.39 | 5.22 | 98.13 ± 2.59 |
| AG II | $Y = 263\,X + 382$ | 1.0000 | 9.03–9030 | 0.86 | 0.38 | 2.35 | 0.21 | 4.96 | 99.51 ± 3.07 |
| IAG II | $Y = 212\,X - 108$ | 1.0000 | 9.73–9730 | 1.50 | 0.16 | 3.88 | 0.37 | 6.01 | 103.27 ± 1.83 |
| AG III | $Y = 188\,X + 656$ | 0.9999 | 9.36–9360 | 0.86 | 0.22 | 1.07 | 0.69 | 3.75 | 96.84 ± 2.91 |
| AG IV | $Y = 42.6\,X + 293$ | 1.0000 | 8.82–8820 | 1.02 | 0.44 | 1.12 | 0.57 | 3.89 | 101.31 ± 1.64 |
| CY | $Y = 149\,X - 175$ | 0.9996 | 8.36–8360 | 1.77 | 0.53 | 3.49 | 0.40 | 4.73 | 104.76 ± 3.29 |
| CAG | $Y = 1640\,X + 14860$ | 1.0000 | 9.55–9550 | 0.11 | 0.29 | 2.77 | 0.73 | 5.15 | 99.02 ± 2.43 |
| ON | $Y = 6410\,X + 32400$ | 1.0000 | 8.45–8450 | 0.08 | 0.58 | 3.02 | 0.65 | 5.86 | 97.87 ± 1.52 |
| ASG | $Y = 9900\,X + 14600$ | 1.0000 | 8.91–8910 | 0.06 | 0.35 | 1.83 | 0.44 | 2.91 | 101.31 ± 1.18 |
| CA | $Y = 22500\,X + 319000$ | 1.0000 | 9.01–9010 | 0.02 | 0.19 | 3.10 | 0.57 | 5.39 | 97.14 ± 2.03 |
| FO | $Y = 63700\,X + 262000$ | 1.0000 | 8.82–8820 | 0.01 | 0.41 | 3.75 | 0.84 | 5.28 | 102.39 ± 1.76 |

[a] The calibration curves were constructed by plotting the peak areas versus the concentration (ng mL$^{-1}$) of each analyte, and each regression equation included eight data points; [b] LOD refers to the limit of detection; [c] RSD (%) = (SD/mean) × 100; [d] Rt refers to the retention time for each analyte in the present LC-MS/MS method; [e] PA refers to the peak area for each analyte in the present LC-MS/MS method; [f] Three different spiking levels (10.57, 21.65 and 30.81 μg g$^{-1}$ for AG I; 11.03, 20.79 and 33.66 μg g$^{-1}$ for AG II; 9.79, 19.92 and 31.04 μg g$^{-1}$ for IAG II; 11.46, 23.39 and 33.51 μg g$^{-1}$ for AG III; 10.84, 20.73 and 29.56 μg g$^{-1}$ for AG IV; 11.57, 23.84 and 33.25 μg g$^{-1}$ for CY; 10.39, 22.55 and 31.26 μg g$^{-1}$ for CAG; 9.61, 22.80 and 30.49 μg g$^{-1}$ for ON; 11.97, 19.63 and 28.46 μg g$^{-1}$ for ASG; 9.33, 20.86 and 30.72 μg g$^{-1}$ for CA; and 10.65, 21.07 and 29.16 μg g$^{-1}$ for FO) were applied in the standard addition recovery study; and the data are presented as the average of three determinations, where standard addition recovery (%) = (amount found – original amount)/amount spiked × 100.

**Table 3.** Analysis of astragalosides and isoflavonoids in different AMHRCs via the developed HSH-CAE-LC-MS/MS method [a].

| AMHRCs types [b] | Contents of astragalosides in AMHRCs (μg $g^{-1}$) [c] | | | | | | Contents of isoflavonoids in AMHRCs (μg $g^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AG I | AG II | IAG II | AG III | AG IV | CY | CAG | ON | ASG | CA | FO |
| AMHRCs I | 927.35 ± 21.37 | 564.61 ± 14.88 | 182.33 ± 9.63 | 162.57 ± 8.52 | 166.72 ± 7.44 | 13.94 ± 1.15 | 6.37 ± 0.41 | 4.79 ± 0.58 | 44.56 ± 5.77 | 65.53 ± 2.28 | 51.89 ± 3.99 |
| AMHRCs II | 1216.83 ± 33.05 | 637.51 ± 17.52 | 171.42 ± 11.23 | 158.69 ± 11.66 | 163.96 ± 9.93 | 15.84 ± 0.94 | 10.52 ± 0.66 | 5.79 ± 0.13 | 68.27 ± 3.72 | 97.26 ± 5.64 | 65.93 ± 1.57 |
| AMHRCs III | 914.22 ± 28.69 | 567.56 ± 23.35 | 185.19 ± 8.45 | 155.84 ± 14.74 | 165.55 ± 13.50 | 14.67 ± 1.33 | 7.58 ± 0.38 | 5.03 ± 0.27 | 44.91 ± 5.40 | 69.84 ± 8.13 | 57.62 ± 5.46 |
| AMHRCs IV | 933.11 ± 19.40 | 571.23 ± 29.88 | 176.87 ± 15.02 | 163.95 ± 13.91 | 168.64 ± 10.67 | 15.33 ± 1.62 | 7.94 ± 0.72 | 4.88 ± 0.50 | 47.32 ± 1.67 | 68.15 ± 5.43 | 53.61 ± 4.02 |
| AMHRCs V | 949.51 ± 32.33 | 593.88 ± 12.79 | 195.03 ± 15.17 | 165.49 ± 7.26 | 158.76 ± 13.89 | 16.77 ± 1.29 | 8.45 ± 0.45 | 5.54 ± 0.22 | 53.82 ± 3.51 | 75.09 ± 4.60 | 58.71 ± 3.15 |
| AMHRCs VI | 1275.39 ± 51.27 | 707.61 ± 25.03 | 203.72 ± 9.01 | 198.55 ± 13.23 | 179.88 ± 16.09 | 17.63 ± 1.87 | 9.51 ± 0.43 | 6.07 ± 0.19 | 60.92 ± 2.53 | 88.73 ± 1.99 | 63.80 ± 3.34 |
| AMHRCs VII | 1231.06 ± 43.94 | 695.82 ± 31.17 | 211.69 ± 16.83 | 180.23 ± 14.19 | 162.46 ± 11.68 | 17.69 ± 2.03 | 9.78 ± 0.96 | 5.93 ± 0.44 | 64.76 ± 4.32 | 91.35 ± 3.85 | 61.62 ± 4.73 |
| AMHRCs VIII | 1184.22 ± 35.52 | 610.75 ± 18.93 | 184.26 ± 10.75 | 151.65 ± 8.33 | 166.43 ± 7.27 | 15.29 ± 1.41 | 6.49 ± 0.63 | 4.18 ± 0.35 | 49.28 ± 2.27 | 63.97 ± 4.41 | 56.35 ± 2.13 |

[a] Operational conditions of HSH-CAE were performed as follows: extraction solvent 80% ethanol, homogenization time 56 s, negative pressure -0.077 MPa, liquid/solid ratio 5.2 and extraction time 19.3 min; [b] Eight candidate AMHRCs (I–VIII) were originated from eight distinct hairy root lines; [c] The contents of analytes were calculated based on dry weights of AMHRCs which were obtained through converting fresh weights by the aid of their moisture contents.

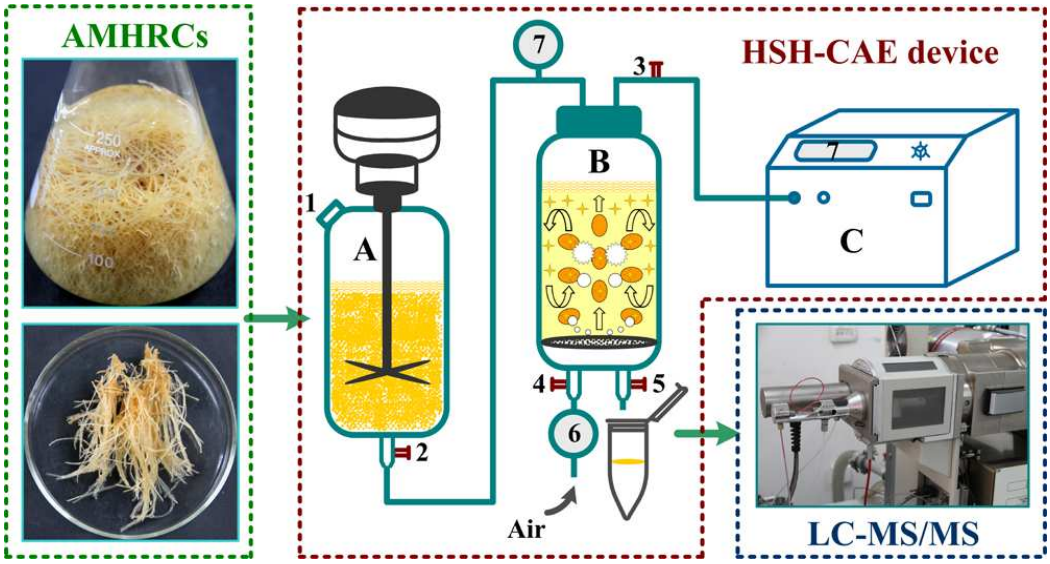


**Fig. 1, Jiao et al.**

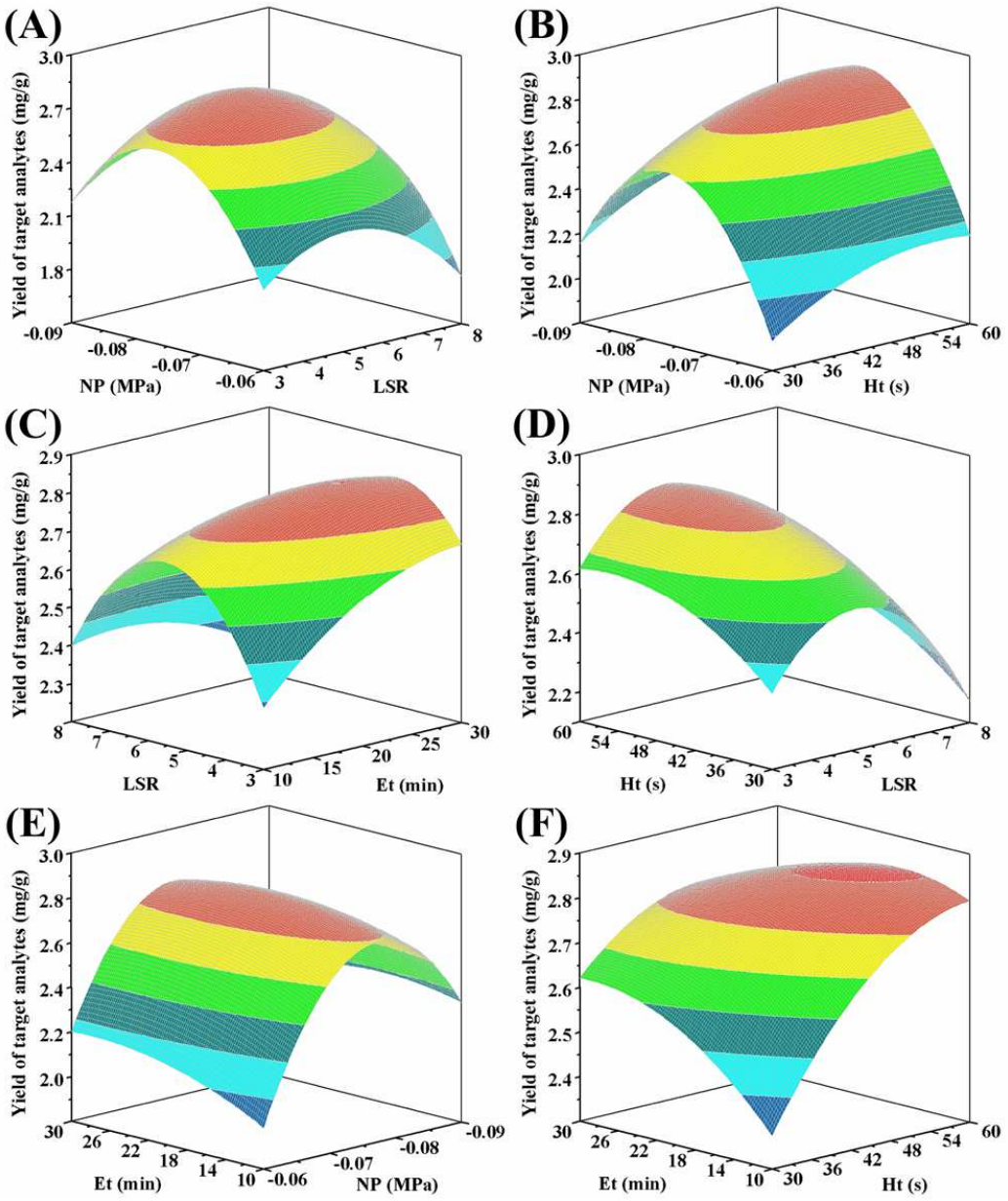


**Fig. 2, Jiao et al.**

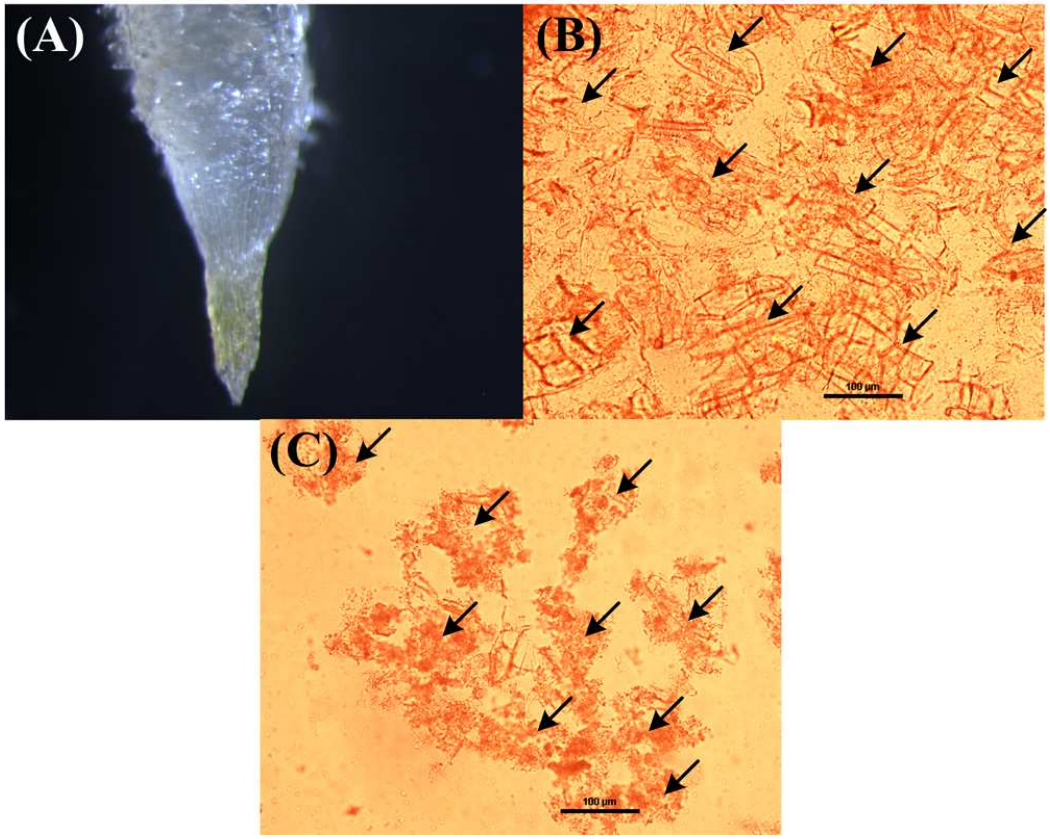


**Fig. 3, Jiao et al.**

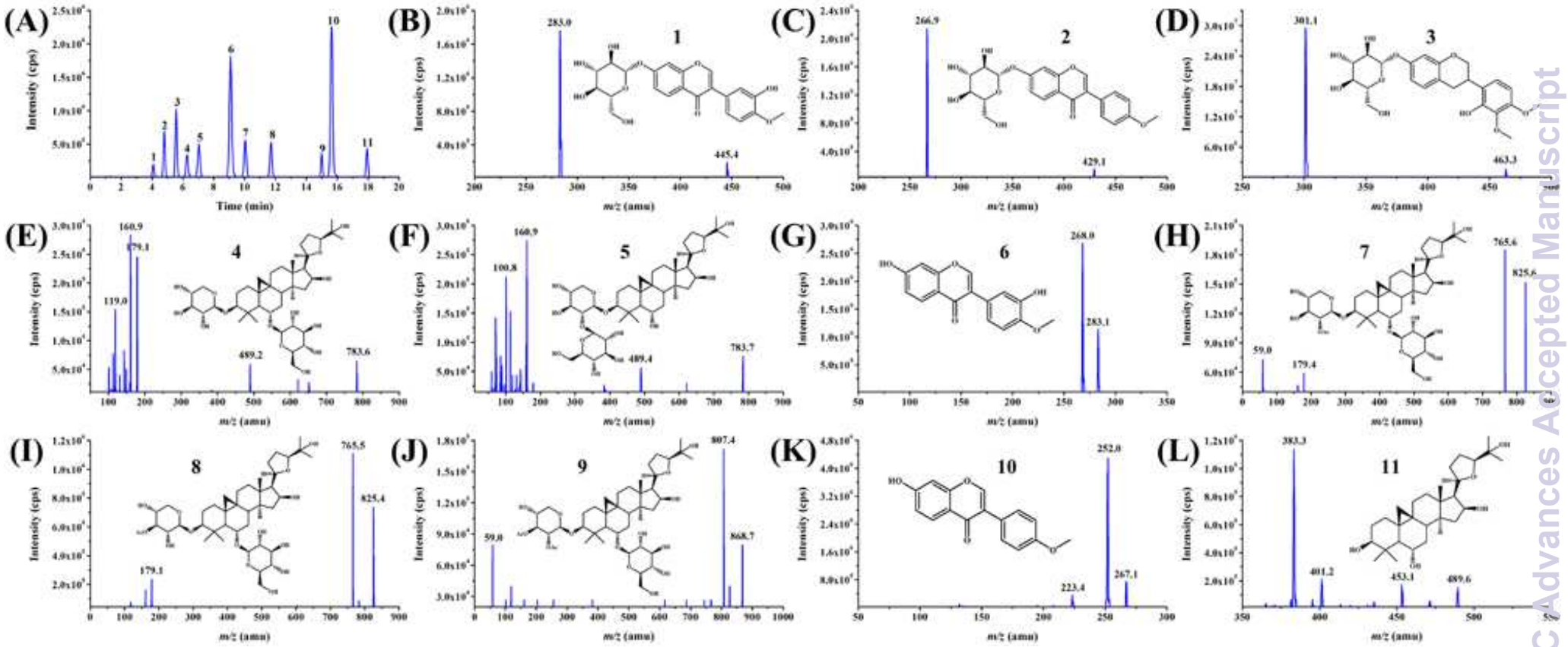


**Fig. 4, Jiao et al.**

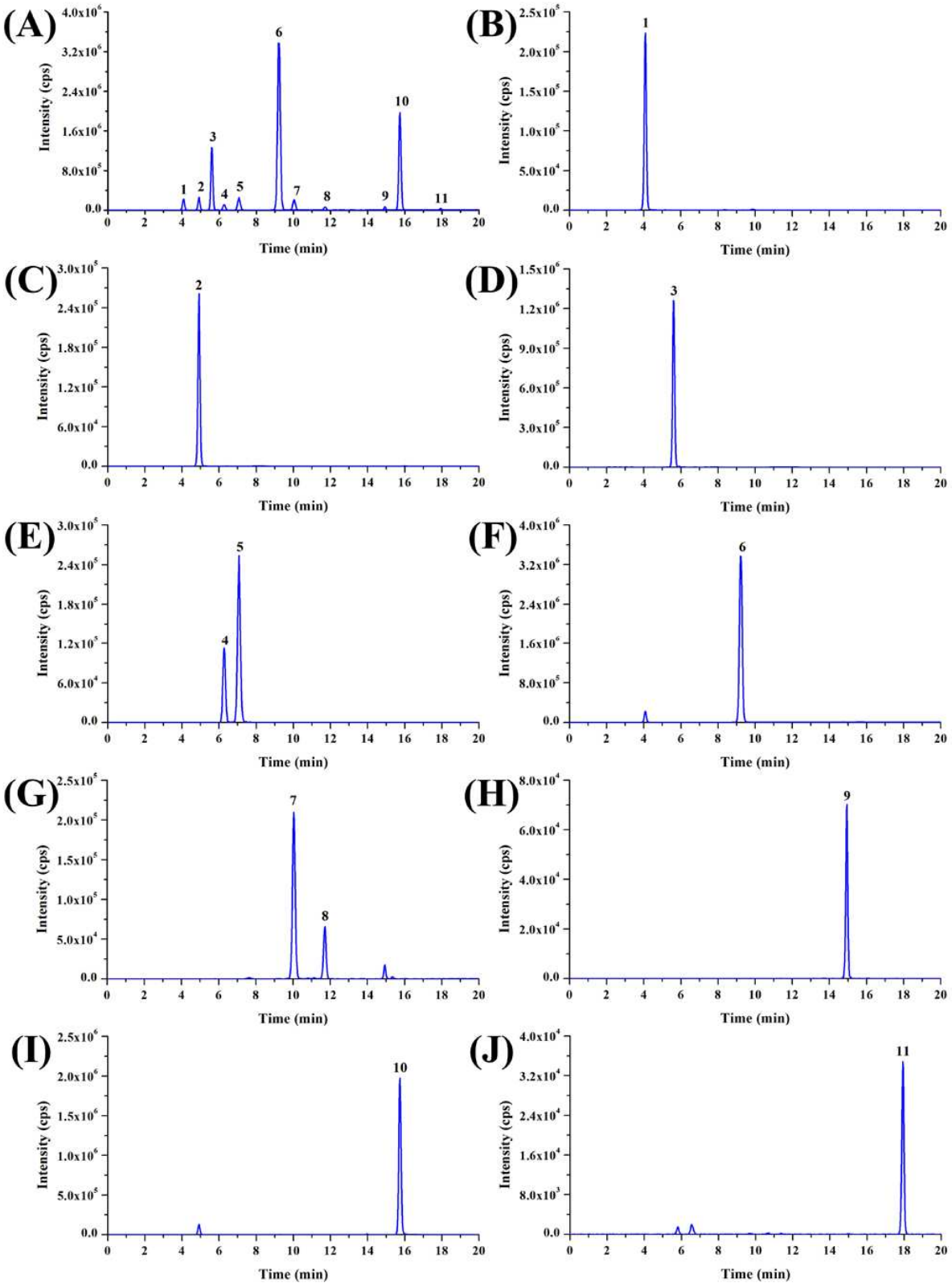


**Fig. 5, Jiao et al.**