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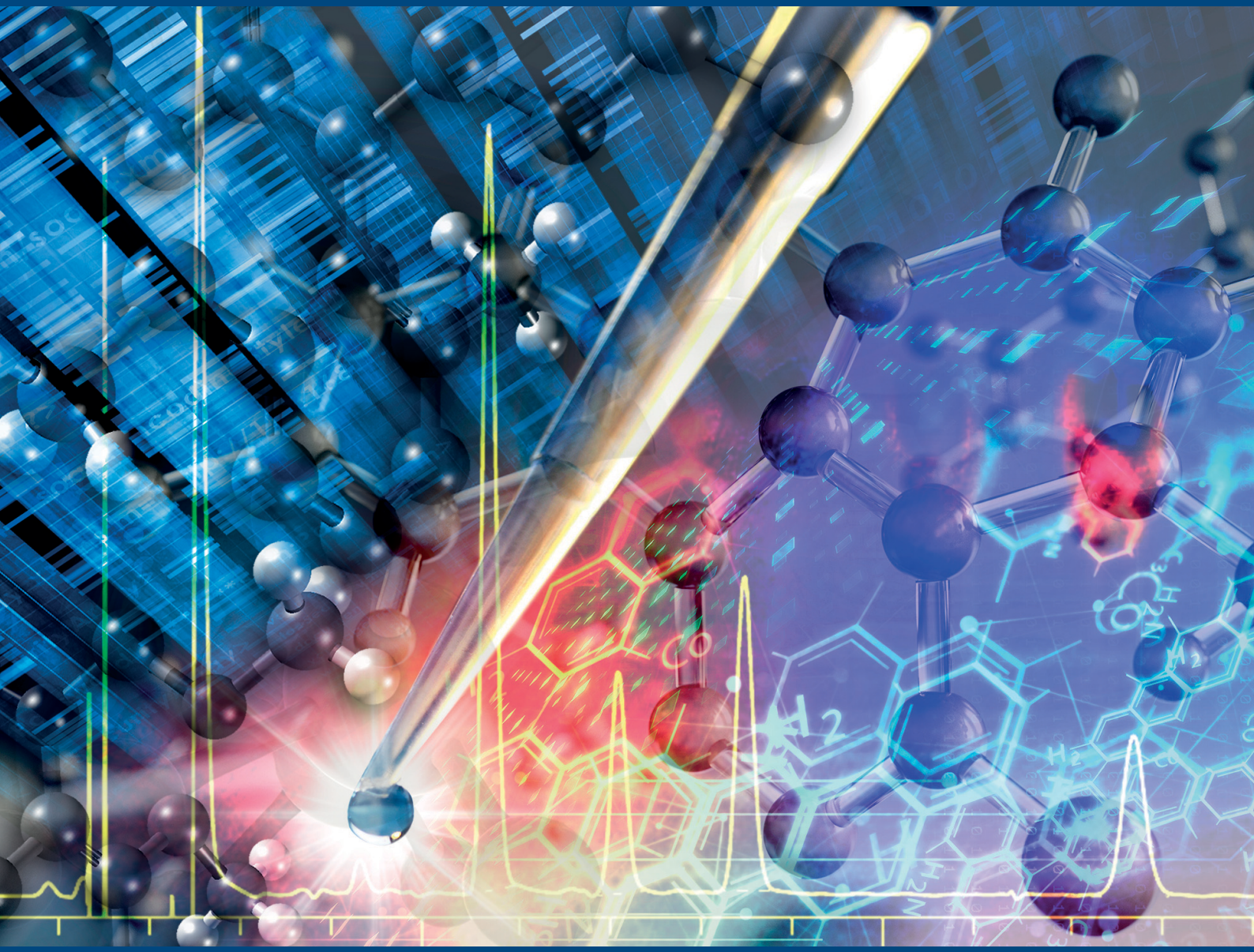

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RESEARCH ARTICLE

Targeted quantitative analysis of monoterpenoid indole alkaloids in *Alstonia scholaris* by ultra-high-performance liquid chromatography coupled with quadrupole time of flight mass spectrometry

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Monoterpene indole alkaloids exhibit structural diversity in herbal resources and have been developed as promising drugs owing to their significant biological activities. Confidential identification and quantification of monoterpene indole alkaloids is the key to quality control of target plants in industrial production but has rarely been reported. In this study, quantitative performance of three data acquisition modes of ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry including full scan, auto-MS^2 and target-MS^2, was evaluated and compared for specificity, sensitivity, linearity, precision, accuracy, and matrix effect using five monoterpene indole alkaloids (scholaricine, 19-*epi*-scholaricine, vallesamine, picrinine, and picralinal). Method validations indicated that target-MS^2 mode showed predominant performance for simultaneous annotation and quantification of analytes, and was then applied to determine monoterpene indole alkaloids in *Alstonia scholaris* (leaves, barks) after extraction procedures optimization using Box-Behnken design of response surface methodology. The variations of *A. scholaris* monoterpene indole alkaloids in different plant parts, harvest periods, and post-handling processes, were subsequently investigated. The results indicated that target-MS^2 mode could improve the quantitative capability of ultra-high-performance liquid

Article Related Abbreviations: AM, accurate mass; BBD, Box-Behnken design; FI, fragment ion; HRMS, high-resolution mass spectrometry; IR, ion ratio; MIA, monoterpene indole alkaloid; Rs, chromatographic resolution; RSM, response surface methodology; RT, retention time.

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chromatography coupled with quadrupole time-of-flight mass spectrometry for structure-complex monoterpene indole alkaloids in herbal matrices.

Alstonia scholaris, monoterpene indole alkaloids, quadrupole time of flight mass spectrometry, qualitative and quantitative analysis, ultra-high-performance liquid chromatography

1 | INTRODUCTION

Monoterpenoid indole alkaloids (MIAs) are a class of secondary metabolites in herbal plants and have become a promising source of new drug discovery because of their outstanding pharmacological activities [1]. Bioactive compounds, such as reserpine (antihypertensive) from *Rauvolfia* species [2], vincamine (nootropic) from the leaves of *Vinca minor* [3], and vinblastine/vincristine (antitumor) from *Catharanthus roseus* [4] are used as important clinical drugs [1, 5]. MIAs have attracted widespread attention from phytochemists and synthetic chemists owing to their structural novelty, complexity, chirality, and diversity resulting from biosynthetic processes. The biosynthesis of MIAs starts from a key intermediate, strictosidine, which is formed by the condensation of tryptophan and secologanin [6, 7]. A series of structure-diverse (more than 40 types) and amount-numerous (over 3000 members) MIAs have been obtained through the diversification of strictosidine by an array of reactions, including oxidation, reduction, methylation, cyclization, and ring-opening, in the biosynthesis of MIAs. To date, the chemical complexity of MIAs has hindered their laboratory synthesis in high yields, resulting in the industrial production of MIAs relying on medicinal plant extraction and isolation. Confidential annotation and quantitative analysis of MIAs in complex herbal matrices are the keys to quality control processes of medicinal plants, which are also essential issues for further investigation of biological functions and toxicological effects. Therefore, confidential annotation and quantification methods are required to support the development of MIAs.

HPLC coupled with a UV detector is a conventional method for the quantitative determination of MIAs. A few methods, including quantitative ^{1}H NMR [8] and HPTLC [2, 9], have also been reported. The accurate identification of MIAs in complex matrices is a prerequisite for quantitative analysis. The challenges in MIAs annotation in complex matrices mainly included co-elution and isomers discrimination. However, HPLC-UV and HPTLC showed poor performance in characterizing co-eluted MIAs, and quantitative ^{1}H NMR made it difficult to identify target analytes in the presence of numerous matrix components. Thus, large deviations in the quantitation results may be caused by misleading qualitative analysis using the above methods because of the numerous co-existing MIA isomers and other metabolites [10, 11]. With advances in analytical technology, HPLC or UHPLC coupled with MS have been increasingly applied for the simultaneous identification and quantification of MIAs in herbal matrices owing to their better selectivity, higher sensitivity, and faster speed [12–14]. Although low or unit resolution triple quadrupole mass spectrometer in selected reaction monitoring or multiple reaction monitoring mode is considered the "gold standard" in the quantitative analysis of small molecules [15, 16], limitations, such as restricted detection number of analytes at the same time and confined selectivity caused by limited resolution, still exist [15, 17]. In recent years, UHPLC coupled with high-resolution MS (HRMS) has triggered new techniques for the quick qualitative and targeted quantitative analysis [18] of phytochemicals [11, 19, 20] and drugs [21, 22]. The limitations in triple quadrupole mass spectrometer detection caused by a unit or low resolution can be compensated by the application of HRMS instruments [17]. In particular, UHPLC-Q-TOF-MS is one of the top HRMS instruments with a series of outstanding performances, including a fast data acquisition rate, superior sensitivity, and the best mass accuracy [23]. UHPLC-Q-TOF-MS records high-resolution full-scan data together with MS^2 fragmentation information in various acquisition modes, enabling concurrent identification together with quantification of pre-known analytes and additional retrospective data mining [16, 18]. Small molecules can be confidentially annotated based on matching orthogonal properties, such as retention time (RT), accurate mass (AM), fragment ions (FIs), and ion ratio (IR), which discriminate between target analytes and interfering matrix components. To the best of our knowledge, several studies have been published on the separation and characterization of specific MIAs via UHPLC-Q-TOF-MS in medicinal plants [2, 24–27]. However, simultaneous annotation and quantification analyses of MIAs are fairly limited. In addition, data acquisition modes may affect the quantitation results, and only one study has reported a comparation of various UHPLC-Q-TOF-MS modes in toxicants quantification

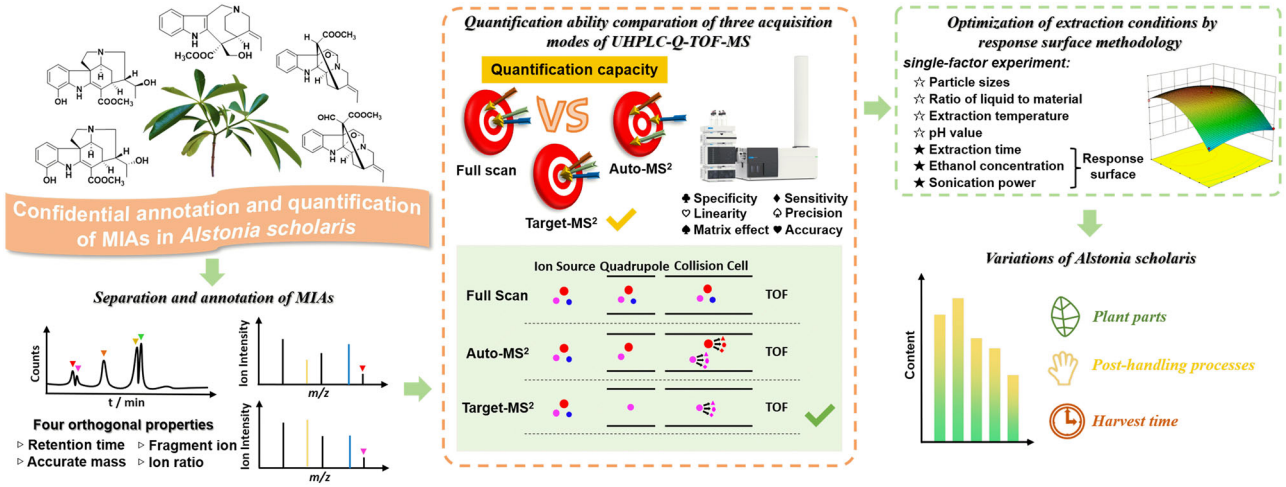

FIGURE 1 A simplified workflow illustrating the UHPLC-Q-TOF-MS for confidential annotation and quantification of monoterpenoid indole alkaloids (MIAs) and application in *Alstonia scholaris* samples.

[21]. Therefore, it is necessary to assess and compare the potential of different UHPLC-Q-TOF-MS modes for MIA analysis in complex herbal matrices.

Alatonia scholaris is distributed widely in South and Southeast Asia [28], whose leaves are used to treat chronic respiratory disease in "dai" ethno-pharmacy historically in the Yunnan province, P. R. China [7]. MIAs have been proven the major active components of *A. scholaris* leaves in our continuing investigations [7, 29, 30]. In this study, five MIAs from *A. scholaris* were taken as examples to investigate the qualitative and quantitative performance of UHPLC-Q-TOF-MS, and the workflow as illustrated in Figure 1. Good separation and accurate identification of MIAs were initially achieved by UHPLC-Q-TOF-MS. Next, the quantification performance of three modes (full scan, auto-MS^2, and target-MS^2) of UHPLC-Q-TOF-MS was compared by specificity, sensitivity, linearity, precision, accuracy, and matrix effect. Afterward, extraction conditions of MIAs from *A. scholaris* leave (main medicinal part) were optimized by Box-Behnken design (BBD) of response surface methodology (RSM). Finally, variations of MIAs in *A. scholaris* with different plant parts, post-handling processes, and harvest time were investigated.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

LC-MS grade methanol, ethanol, ACN, and formic acid were purchased from Merck Company (Darmstadt, Germany). Distilled water was procured from the specialty store of Watsons. The five standard MIA compounds including scholaricine, 19-*epi*-scholaricine, vallesamine, picrinine, and picralinal were isolated, purified, and identified from *A. scholaris* by our lab before [31], with a purity of scholaricine and 19-*epi*-scholaricine > 98 %, and vallesamine, picrinine, and picralinal > 95 %.

All the test samples were collected from two plantlets of *A. scholaris*, cultivated at Yunnan University campus. A total of twenty *A. scholaris* samples from different plant parts, post-handling processes, and harvest times were collected, whose related information was shown in Tables S1–S3, respectively. Plant samples were immediately oven-dried at 50°C to constant weight after harvest. The dried plant samples were further pulverized with a grinder, sieved into fine powders, and stored in a light-tight container with silica gel desiccant at room temperature for further analysis.

2.2 | LC-MS condition settings and optimization

An Agilent 1290 Infinity II ultra high-performance liquid chromatograph coupled with an Agilent G6545B quadrupole time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a dual AJS ESI source system was employed. A reverse column (an SB-C18 column, 2.1 × 100 mm, 1.8 μm, Agilent Technologies) was employed to separate MIAs in *A. scholaris*. A sample volume of 0.5 μl was loaded by the autosampler. Eluent A (0.1 % formic acid in water) and eluent B (ACN) were used to achieve efficient separation by gradient elution: 0–7 min, 4%–12 % B; 7–12 min, 12%–17% B; 12–16 min, 17% B; 16–23 min, 17%–30% B; 23–28 min, 30%–45% B; 28–30 min, 45%–65% B; 30.01–35 min, 98% B; 35–40 min, 98%–4% B. Separation conditions

TABLE 1 Analytical conditions of a full scan, auto-MS^2, and target-MS^2 modes of UHPLC-Q-TOF-MS for five monoterpenoid indole alkaloids (MIAs).

Analytes	Full scan	Auto-MS^2		Target-MS^2				
	Extracting ion (*m/z*) $[M+H]^+$	Time segment (min)	Collision energy (eV)	Precursor ion (m/z) $[M+H]^+$	Extracting product ion (*m/z*)	Retention time (min)	Delta Retention time (min)	Collision energy (eV)
Scholaricine	357.18144	0–15	21	357.18144	325.15591	13.0	5.0	21
19-*epi*-scholaricine	357.18144	0–15	21	357.18144	325.15591	13.0	5.0	21
Vallesamine	341.18656	15–22	15	341.18656	282.14879	18.5	5.0	15
Picrinine	339.16999	22–45	26	339.16999	106.06541	24.7	3.0	27
Picralinal	367.16489	22–45	26	367.16489	106.06541	25.3	3.0	25

containing column temperature and flow rate were maintained at 35°C and 0.15 ml/min, respectively.

The ion source was operated with the following parameters under positive ion mode: gas temperature 325°C; drying gas flow rate 9 L/min; nebulizer pressure 35 psi; sheath gas temperature 365°C; sheath gas flow rate 11 L/min; nozzle voltage 500 V; VCap voltage 3500 V; Oct1 RF Vpp 750 V; fragmentor voltage 115 V; skimmer voltage 65 V. MS^1 and MS^2 acquisition rates were 3 and 4 spectra/s, respectively. Both MS^1 and MS^2 data were collected from 20 to 1000 Da. Data acquisition modes of UHPLC-Q-TOF-MS included full scan, auto-MS^2, and target-MS^2, and key parameters of three modes were rigorously optimized (experiment details were provided in Note S1 and the results are shown in Table 1). For auto-MS^2, other common parameters were as follows: precursor threshold 1000 counts; 2 max precursors per cycle; enabling active exclusion.

2.3 | Quantitation method validation and comparation of three modes

The quantitation performance of full scan, auto-MS^2, and target-MS^2 acquisition modes was validated and compared according to the ICH guideline [32], and partially on the AOAC [33], EMA [34], and FDA [35] guidelines, which included specificity, sensitivity, linearity, precision, accuracy, and matrix effect validation. The experimental details of quantitation method validation were presented in Note S2.

2.4 | Experimental design of RSM

The influence of seven separate and essential factors on the total extraction yield of five MIAs (total peak area was used to calculate the total extraction yield of five MIAs) from *A. scholaris* was initially evaluated through single-factor experiments. Next, three significant factors including ethanol concentration (X_1), extraction time (X_2), and sonication power (X_3) were selected as independent variables, and the total peak area of five MIAs (Y) was employed as the response value to conduct RSM analysis based on the BBD with three variables and three levels. An empiric second-order polynomial model was constructed to describe the mathematical relationship between independent variables and response value according to the results of seventeen orthogonal experiments, and analysis of variance was employed to evaluate the adequacy of the RSM model by lack-of-fit, R^2, *p*-test, and *F*-test value, and so forth. The RSM model was illustrated as contour plots and 3D-surface. Finally, the theoretical optimal extraction conditions and response value were verified through the experiment. Further details on the RSM experiment and analysis were presented in Note S3.

2.5 | Plant samples preparation

The dried powder of each *A. scholaris* sample was accurately weighed (20.00 mg, 100-mesh). Next, 1 ml 57 % ethanol solvent was added to extract MIAs by ultrasonic-assisted extraction in a water bath (50°C) for 9 min with 64 % sonication power. After that, the extracts were centrifuged at 13 000 rpm for 15 min at 4°C, and the supernatant was filtered through a 0.22 μm polytetrafluoroethylene filter and diluted 10-fold with 57 % ethanol solvent for further UHPLC-Q-TOF-MS analysis. Three replicates were prepared per sample and the results were shown as "mean ± SD".

2.6 | Statistics

UHPLC-Q-TOF-MS data were processed using Agilent MassHunter Qualitative Analysis Navigator B.08.00

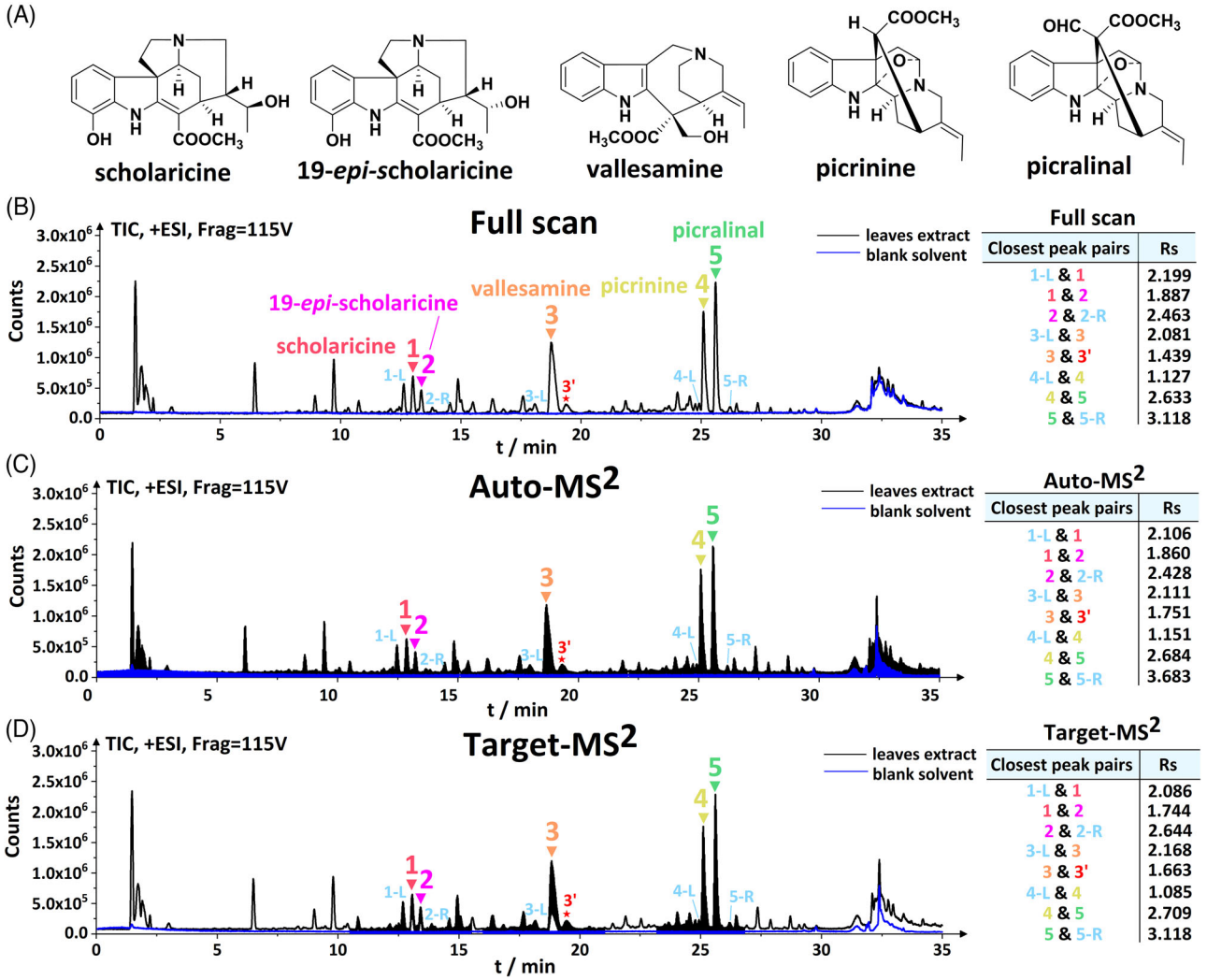

FIGURE 2 Chemical structures of five monoterpenoid indole alkaloids (MIAs) (A); Total ion chromatograms (TICs) and chromatographic resolution (Rs) of *Alstonia scholaris* leaf extracts in full scan (B), auto-MS^2 (C), and target-MS^2 (D) modes.

software. RSM model was designed and analyzed by Design Expert software (Trial Version 10.0.3, Stat–Ease Inc., Minneapolis, MN, USA).

3 | RESULTS AND DISCUSSION

3.1 | Monoterpene indole alkaloids identification and discrimination

Discrimination of co-eluted MIAs and isomers was the key to MIAs identification in complex matrices. Figure 2 illustrated the chemical structures of five MIAs and typical total ion chromatograms of *A. scholaris* leaf extracts in full scan, auto-MS^2, and target-MS^2 modes. Full scan mode annotated MIAs from only two properties (RT + AM), which was inadequate for the distinguishment of isomers. Auto-MS^2 and target-MS^2 identified MIAs from four orthogonal properties (RT + AM + FI + IR), which enabled precise characterization of MIA isomers. As shown in Figure 3, two pairs of isomers with *m/z* 357.1804 and 341.1858, were taken as examples to illustrate how auto-MS^2 and target-MS^2 modes annotated MIAs through RT + AM + FI + IR. Scholaricine (peak 1) and 19-*epi*-scholaricine (peak 2) were a pair of isomers with the same planner structure and different absolute configurations of C-19 (scholaricine: *R*; 19-*epi*-scholaricine: *S*), which caused almost same MS^2 spectra, but could be accurately distinguished by a weak FI *m/z* 307.1438 ($[M\text{-}CH_4O\text{-}H_2O+H]^+$) in MS^2 spectrum of 19-*epi*-scholaricine (Figure 3A). Besides, consistent differences in IR from *m/z* 357.1804 to *m/z* 325.1550 under different CEs could also be used to distinguish scholaricine and 19-*epi*-scholaricine (Figure 3A). Vallesamine (peak 3) and its co-eluted peak (peak 3′) were a pair of isomers with

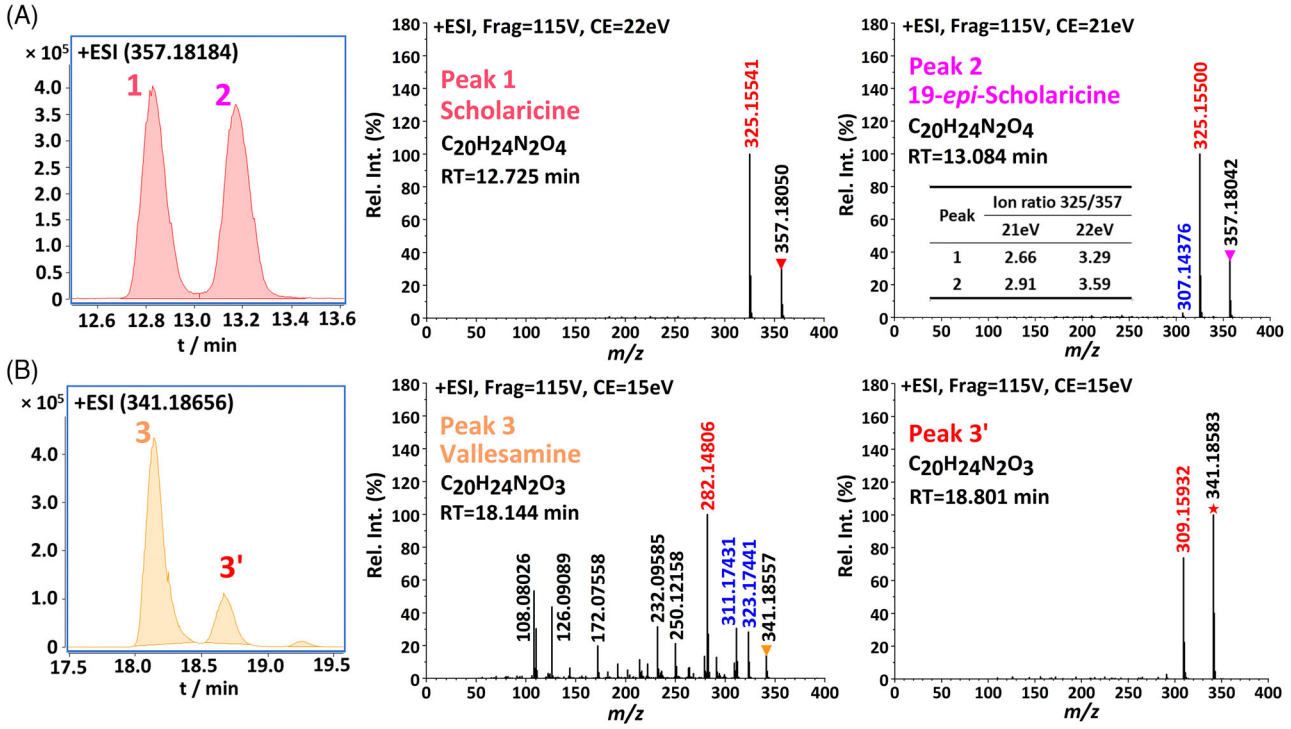

FIGURE 3 Annotation of MIA isomers: extracted ion chromatogram (EIC) of full scan mode and MS^2 spectra of isomers with *m/z* 357.1805 (A) and *m/z* 341.1855 (B).

m/z 341.1858, which could be easily distinguished by FIs under the same CE (Figure 3B). Thus, compared with full scan mode, auto-MS^2, and target-MS^2 modes could accurately identify MIAs through RT + AM + FI + IR, which provided the basis for accurate quantitation.

3.2 | Quantitative performance comparation of three data acquisition modes

3.2.1 | Specificity (selectivity)

Specificity (selectivity) is understood as the capacity of which a method can identify the analyte accurately in the presence of interferents (such as complex components in plant matrices). Total ion chromatograms of *A. scholaris* leaf extracts in three modes were exhibited in Figure 2 to illustrate specificity, in accordance with the ICH guideline [32]. Besides, according to the AOAC guideline [33], chromatographic resolution (Rs) between target peaks and their closest peaks were calculated, and Rs value more than 1 indicated the good separation of three modes (Rs of full scan: 1.127–3.118; Rs of auto-MS^2: 1.151–3.683; Rs of target-MS^2: 1.085 – 3.118) (Figure 2).

Compared to extracted ion chromatograms of five MIAs in three modes (Figure S2), many isomer peaks of target analytes were observed in full scan mode due to only precursor ion mass extraction but rarely observed in auto-MS^2 and target-MS^2 mode because of specific ion pairs extraction (precursor ion → product ion). Compared extracted ion chromatogram peak shape of the three modes (Figure S2), target-MS^2 played better than auto-MS^2, which may be caused by the ion-selected mechanism of the quadrupole as described in Note S1. In auto-MS^2 mode, an abundance of some interfering ions such as background or co-eluted ions was probably stronger than the target ion at the front or the end of target peak separation, which may lead to MS^2 spectra loss of target analytes at the bottom of the peak. In brief, compared with the other two modes, the specificity of target-MS^2 was outstanding.

3.2.2 | Linearity

The linearity of five MIAs in three modes was also investigated and compared. The slope and y-intercept of calibration curves, linearity ranges, and correlation coefficient (R^2) were displayed in Table 2. In the full scan mode, each analyte determined the widest linear ranges (25–50 times) and had the best linear regression ($R^2 > 0.99$). For target-MS^2 mode, shcholaricine and 19-*epi*-scholaricine had good linear regression with narrower linear ranges at 0.800–20.000 μg/ml and 1.000–20.000 μg/ml,

TABLE 2 Linearity comparation of three quantitative modes.

Analytes	Calibration curve			Linear range (μg/ml)			R^2		
	Full scan	Auto-MS^2	Target-MS^2	Full scan	Auto-MS^2	Target-MS^2	Full scan	Auto-MS^2	Target-MS^2
Scholaricine	y = 351514.87x + 689475.27	y = 101597.67x + 276345.33	y = 102721.32x + 284347.38	0.400 –20.000	0.800 –20.000	0.800 –20.000	0.99396	0.99017	0.99169
19-*epi*-scholaricine	y = 294618.47x + 926572.28	y = 82875.10x + 391284.76	y = 83984.07x + 386919.12	0.800 –20.000	1.000 –20.000	1.000 –20.000	0.99219	0.97482	0.98159
Vallesamine	y = 162832.54x + 1192473.80	y = 15646.27x + 175399.34	y = 15202.99x + 111050.82	1.264 –63.200	3.160 –63.200	1.264 –63.200	0.99616	0.99062	0.99527
Picrinine	y = 892304.00x + 738032.82	y = 43399.14x + 68356.12	y = 43257.74x + 49011.81	0.358 –17.880	0.715 –17.880	0.358 –17.880	0.99934	0.99165	0.99791
Picralinal	y = 669595.85x + 658429.22	y = 26941.59x + 31353.08	y = 26387.30x + 34412.10	0.349 –17.440	0.349 –17.440	0.349 –17.440	0.99911	0.99603	0.99733

while linear range and regression of vallesamine, picrinine, and picralinal was as good as full scan mode. In auto-MS^2 mode, for the majority of analytes, good linear regression was achieved only through the narrowest linear range, except for picralinal. Thus, full scan, target-MS^2, and auto-MS^2 had the best, medium, and worst linearity.

3.2.3 | Sensitivity

The LOD and LOQ values of five MIAs in three quantification modes were summarized in Table 3. From the overall data, LOD and LOQ values of auto-MS^2 were highest at 0.648–5.349 and 1.964–16.208 μg/ml, respectively, except for compounds scholaricine and picralinal (whose LOD and LOQ were a little lower than the other two modes). Compared full scan with target-MS^2 mode, although LOD and LOQ value of 19-*epi*-scholaricine was higher in target-MS^2 mode, LOD and LOQ values of picrinine and picralinal were equal in the two modes, while scholaricine and vallesamine determined by the target-MS^2 had 88 – 96.2 % lower LODs and 88 – 95.7 % lower LOQs than those obtained by full scan mode. The results indicated that the sensitivity of target-MS^2 was a little bit better than full scan and clearly better than auto-MS^2.

3.2.4 | Precision

Precision is defined as the closeness or scatter degree of parallel test results, which is reported as the RSD (%) of intra- and inter-day repeatability at three concentration levels (Table S5) according to the ICH guideline [32]. In this study, intra-day precision showed RSD values of 0.158%–6.833%, 0.213%–14.107%, and 0.268%– 4.145% with full scan, auto-MS^2, and target-MS^2, respectively, while RSD values of inter-day precision in full scan, auto-MS^2, and target-MS^2 were 1.223%–6.024%, 1.215%–16.127%, and 0.978%–4.059%, respectively. Compared with the other two modes, auto-MS^2 mode had the worst performance, which may be caused by the selection uncertainty of precursor ion at the front and end of analytes separation (briefly discussed in Note S1). Both intra- and inter-day precision of target-MS^2 were as good as the full scan mode.

3.2.5 | Accuracy

Accuracy describes the closeness of measured value by the developed method to the pre-known true value, which

TABLE 3 Sensitivity comparation of three quantitative modes.

Analytes	LOD (μg/ml)			LOQ (μg/ml)		
	Full scan	Auto-MS^2	Target-MS^2	Full scan	Auto-MS^2	Target-MS^2
Scholaricine	0.053	0.648	0.002	0.161	1.964	0.007
19-*epi*-scholaricine	0.016	0.243	0.457	0.049	0.735	1.386
Vallesamine	0.330	5.349	0.040	1.001	16.208	0.120
Picrinine	0.152	1.135	0.181	0.460	3.439	0.547
Picralinal	0.098	0.020	0.106	0.296	0.059	0.321

was assessed by spiked recovery (%). In this study, the accuracy of scholaricine, picrinine, and picralinal was normal with spiked recoveries at 90.47%–109.41%, 75.49%–124.555%, and 80.37%–108.747% in full scan, auto-MS^2, and target-MS^2 mode, respectively (Table S6). Spiked recoveries of 19-*epi*-scholaricine and vallesamine were bigger and smaller than the normal range, which may be caused by the matrix effect or molecular properties (Table S6). The results indicated that full scan, target-MS^2, and auto-MS^2 had the best, medium, and worst accuracy.

3.2.6 | Matrix effect

The matrix effect is the alternation in analyte response with the presence of interfering substances in matrices, which was evaluated by the slope comparison method in this study. The results of the matrix effect were presented in Table S7. The slope ratio value of scholaricine, picrinine, and picralinal was close to 1.0, which indicated they were not significantly affected by matrix components in the three modes. However, the slope ratio of 19-*epi*-scholaricine (increased) and vallesamine (decreased) indicated that they were affected by matrices by ionization enhancement and suppression, respectively, which could be the reason for spiked recovery change of 19-*epi*-scholaricine and vallesamine in accuracy test. The slope ratio of three modes was compared through three MIAs without significant matrix effect (Table S7), and the results indicated that full scan, target-MS^2, and auto-MS^2 had the best, medium, and worst matrix effects.

Together these quantitative method validation results indicated that target-MS^2 mode was the optimal acquisition mode for MIAs identification and quantification in complex herbal matrices because of its best performance (outstanding specificity; best sensitivity and precision; good linearity, accuracy, and matrix effect).

3.3 | Optimization of extraction conditions by RSM

RSM is a widely used statistical tool for process optimization through orthogonal experiments designing, mathematical model constructing, and statistical results analysis. In this study, the empiric second-order polynomial model describing the relationship between the response value and three independent variables was expressed by the following equation, which was derived from the BBD of RSM with three variables and three levels:

$$\begin{aligned} Y = (&79.700 - 8.341X_1 - 1.023X_2 - 0.433X_3 \\ &+ 0.747X_1X_2 + 2.623X_1X_3 - 0.172X_2X_3 \\ &- 10.420X_1^2 - 3.669X_2^2 - 3.803X_3^2) \times 10^6 \end{aligned}$$

where Y is the total peak area of five MIAs; X_1, X_2, and X_3 are ethanol concentration, extraction time, and sonication power, respectively.

The results of analysis of variance analysis were shown in Table S17. In this model, a p-value < 0.001 (high significance) indicated model-suitable. The p-values of variables < 0.001, < 0.01, or > 0.05 were defined as "very significant", "significant", or "not significant", respectively. A high determination coefficient ($R^2 = 0.9558$) proved that the model could explain 95.58 % of the response changes. The p-value of the lack-of-fit ($p = 0.2911 > 0.05$, insignificant) indicated the adequacy of the fit. An adequate precision value of 11.376 (>4) indicated proper model discrimination. Thus, the above results demonstrated that the model can simulate the relationship between the response value and the three variables.

Contour plots and 3D surfaces of RSM were illustrated in Figure 4, which showed the influence of every two variables in the total peak area of five MIAs. The theoretical optimal extraction conditions were as follows: ethanol concentration of 57.044 % (v/v), extraction time of 9.108 min, sonication power of 63.924%; the theoretical

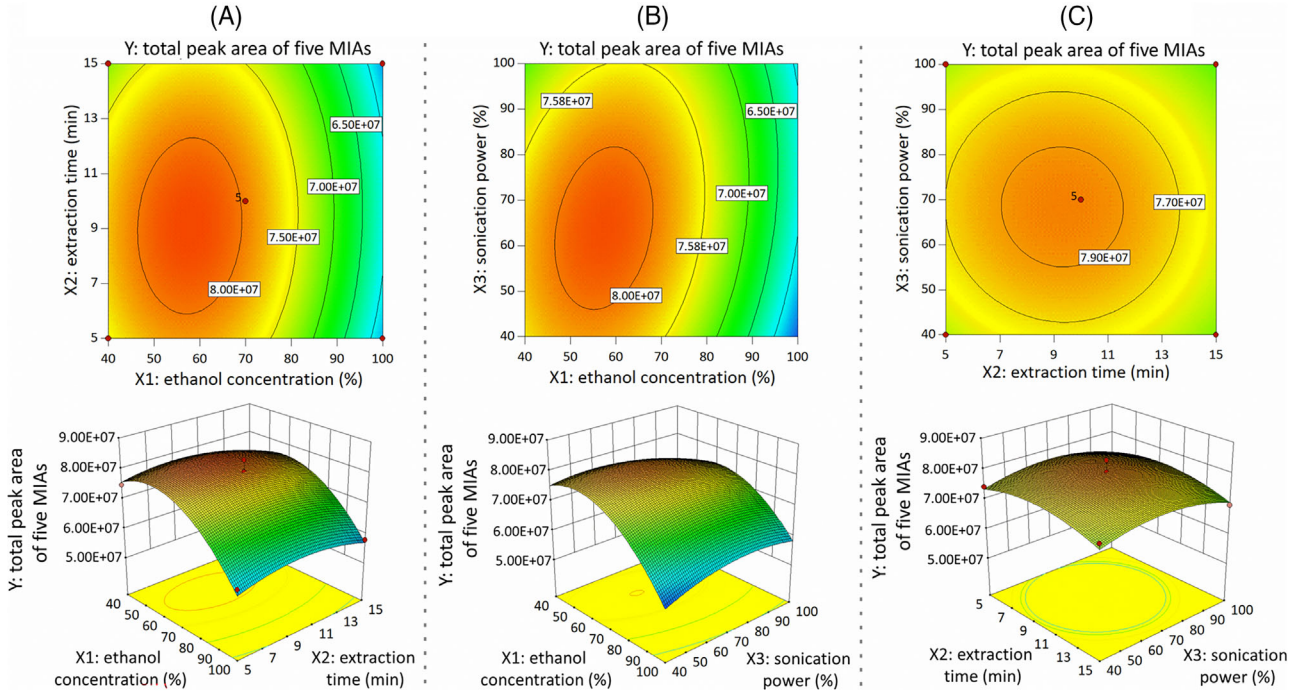

FIGURE 4 Contour plots and response surface (3D) showed the effect of ethanol concentration (X_1), extraction time (X_2), and sonication power (X_3) on the total peak area of five monoterpenoid indole alkaloids (MIAs) (Y).

maximum total peak area of five MIAs was 8.163×10^7. The experimental total peak area of five MIAs under optimal conditions was 7.805×10^7 ($n = 5$) with the bias of the practical value to the predicted one lower than 5%, which verified the adequateness of the model.

3.4 | Quantitative results of various *A. scholaris* samples by target-MS^2 acquisition mode

In this study, target-MS^2 mode and optimal extraction conditions were applied to analyze five MIAs in various *A. scholaris* samples with different plant parts, post-handling processes, and harvest time. Picrinine, picralinal, and scholaricine with weak matrix effect were quantified using the external standard method; vallesamine and 19-*epi*-scholaricine with severe matrix effect were quantified through standard addition method as a recommendation of the previous report [36]. The quantitative results were listed in Table S18 and illustrated in Figure 5.

Content distribution of five MIAs in different plant parts were shown in Figure 5A. MIAs were mainly distributed in young barks, mature barks, and leaves. There were almost no MIAs in old barks. The content of MIAs in young, mature, and old leaves had no significant differences, which indicated that leaves at different growth periods could be harvested.

Content differences of five MIAs in *A. scholaris* leaves with different post-handling processes were illustrated in Figure 5B. Compared with the shade-dried process, a significant decrease of MIAs contents post-handled by the sun-dried process was observed, which may be caused by analyte degradation due to high-energy rays in sunlight. Shade-dried for about 8 days didn't decrease the total content of five MIAs significantly.

Monthly content variations of five MIAs in *A. scholaris* leaves from September 2020 to April 2021 were shown in Figure 5C. The content increased from September to October and peaked in October, and then decreased from October to December probably due to the low temperature of winter. Afterward, content increased overall with the weather and climate warming from January to April. The slight decrease in content in March may be caused by a cold spell in later spring. Overall, the total content of five MIAs had no significant differences from September to April.

4 | CONCLUDING REMARKS

The challenges in MIAs annotation in *A. scholaris* matrices, containing co-eluted MIAs and isomers distinguishment, could be overcome by UHPLC-Q-TOF-MS based on four properties, including RT, AM, FI, and IR, which enabled the accurate quantitation of MIAs. The quantitative performances of three modes, including full scan,

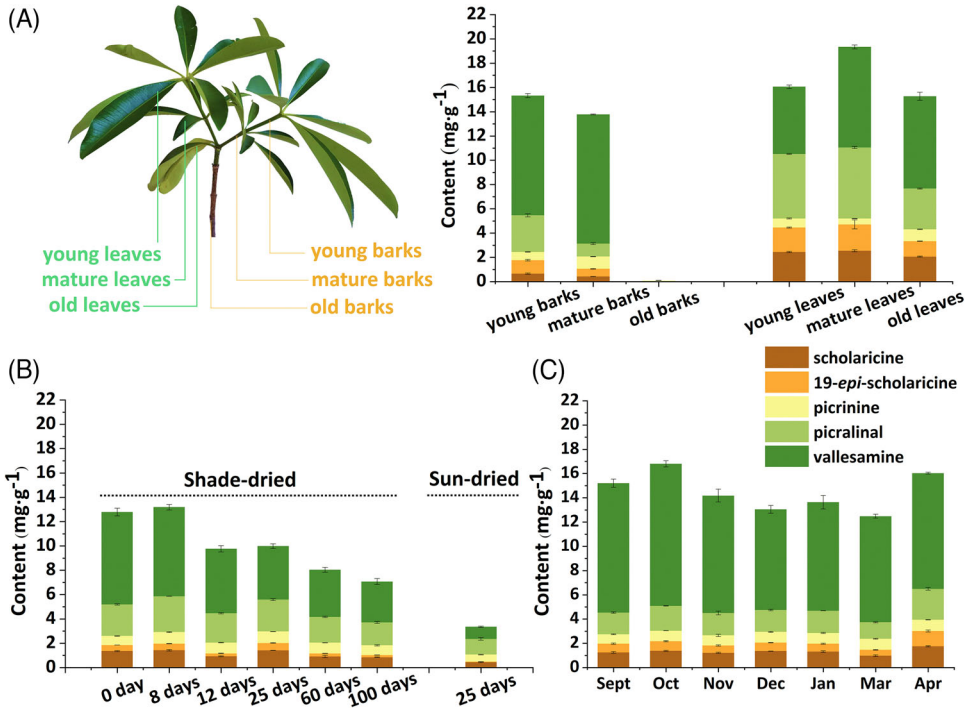

FIGURE 5 Quantitative results of five monoterpenoid indole alkaloids (MIAs) in *Alstonia scholaris* samples with different plant parts (A), post-handling processes (B), and harvest time (C).

auto-MS^2 and, target-MS^2 of UHPLC-Q-TOF-MS were compared, and target-MS^2 was proved the best mode for MIAs quantification owing to its good specificity, sensitivity, precision, linearity, accuracy, and matrix effect. Moreover, extraction conditions were optimized to achieve the maximum total extraction yield of five MIAs. Finally, target-MS^2 was applied to evaluate variations of MIAs in *A. scholaris* samples with different plant parts, post-handling processes, and harvest time under optimal extraction conditions. Quantitative results indicated that: 1) MIAs content differences were not significant in young, mature, and old leaves; 2) leaves post-handling in the shade-dried process retained more MIAs than the sun-dried process; 3) monthly variations of MIAs in *A. scholaris* leaves were not significant. In summary, UHPLC-Q-TOF-MS was available to simultaneously annotate and quantify structure-complex MIAs in herbal matrices.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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REFERENCES

1. Liu XY, Qin Y. Indole alkaloid synthesis facilitated by photoredox catalytic radical cascade reactions. Acc Chem Res. 2019;52(7):1877–91.
2. Bindu S, Rameshkumar KB, Kumar B, Singh A, Anilkumar C. Distribution of reserpine in *Rauvolfia* species from India – HPTLC and LC–MS studies. Ind Crop Prod. 2014;62:430–6.
3. Bonandi E, Foschi F, Marucci C, Paladino G, Luzzani M, Passarella D. Vincamine by synthesis and semi-synthesis. Phytochem Rev. 2020;20(1):343–65.
4. Liu Y, Patra B, Pattanaik S, Wang Y, Yuan L. Gata and phytochrome interacting factor transcription factors regulate light-induced vindoline biosynthesis in *Catharanthus roseus*. Plant Physiol. 2019;180(3):1336–50.
5. Zhou J, Du SY, Dong HJ, Fang L, Feng JH. Preparative separation of monoterpenoid indole alkaloid epimers from *Ervatamia yunnanensis* Tsiang by pH-zone-refining counter-current chro-

matography combined with preparative high-performance liquid chromatography. Molecules 2019;24(7):1316.
6. O'Connor SE, Maresh JJ. Chemistry and biology of monoterpene indole alkaloid biosynthesis. Nat Prod Rep. 2006;23(4):532–7.
7. Cai XH, Du ZZ, Luo XD. Unique monoterpenoid indole alkaloids from *Alstonia scholaris*. Org Lett 2007;9(9):1817–20.
8. Yin T, Lu J, Liu Q, Zhu G, Zhang W, Jiang Z. Validated quantitative ^{1}H NMR method for simultaneous quantification of indole alkaloids in *Uncaria rhynchophylla*. ACS Omega. 2021;6(47):31810–7.
9. Irshad S, Khatoon S. A validated HPTLC method for the simultaneous determination of seasonal alterations of two antihypertensive monoterpenoid indole alkaloids in *Rauvolfia* species from northern India. S Afr J Bot 2021;142:193–200.
10. Chen Q, Zhang W, Zhang Y, Chen J, Chen Z. Identification and quantification of active alkaloids in *Catharanthus roseus* by liquid chromatography-ion trap mass spectrometry. Food Chem 2013;139(1-4):845–52.
11. Li XY, Xu JD, Zhou SS, Kong M, Xu YY, Zou YT, et al. Time segment scanning-based quasi-multiple reaction monitoring mode by ultra-performance liquid chromatography coupled with quadrupole/time-of-flight mass spectrometry for quantitative determination of herbal medicines: *Moutan cortex*, a case study. J Chromatogr A. 2018;1581–2:33–42.
12. Kumar S, Singh A, Bajpai V, Srivastava M, Singh BP, Ojha S, et al. Simultaneous determination of bioactive monoterpene indole alkaloids in ethanolic extract of seven *Rauvolfia* species using UHPLC with hybrid triple quadrupole linear ion trap mass spectrometry. Phytochem Anal 2016;27(5):296–303.
13. Liu J, Liu Y, Pan YJ, Zu YG, Tang ZH. Determination of alkaloids in *Catharanthus roseus* and *Vinca minor* by high-performance liquid chromatography-tandem mass spectrometry. Anal Lett 2015;49(8):1143–53.
14. Kumar S, Singh A, Kumar B, Singh B, Bahadur L, Lal M. Simultaneous quantitative determination of bioactive terpene indole alkaloids in ethanolic extracts of *Catharanthus roseus* (L.) G. Don by ultra high performance liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal 2018;151:32–41.
15. Xue J, Derks RJE, Webb B, Billings EM, Aisporna A, Giera M, et al. Single quadrupole multiple fragment ion monitoring quantitative mass spectrometry. Anal Chem 2021;93(31):10879–89.
16. Gray N, Lawler NG, Yang R, Morillon AC, Gay MCL, Bong SH, et al. A simultaneous exploratory and quantitative amino acid and biogenic amine metabolic profiling platform for rapid disease phenotyping via UPLC-QTOF-MS. Talanta 2021;223(Pt 2):121872.
17. Lindemann V, Schmidt J, Cramer B, Humpf HU, Detection of mycotoxins in highly matrix-loaded house-dust samples by QTOF-HRMS, IM-QTOF-HRMS, and TQMS: advantages and disadvantages. Anal Chem 2022;94(10):4209–17.
18. Cavaliere C, Antonelli M, Capriotti AL, La Barbera G, Montone CM, Piovesana S, et al. A triple quadrupole and a hybrid quadrupole orbitrap mass spectrometer in comparison for polyphenol quantitation. J Agric Food Chem 2019;67(17):4885–96.
19. Gupta BD, Kar A, Narayan S, Thakur CP, Mukherjee PK, Haldar PK. Ultra-performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry-based metabolite profiling, quality evaluation, and marker analysis of *Trachyspermum ammi* (L.) Sprague by high-performance thin-layer chromatography. J Sep Sci 2023;46:2200872.
20. Deng J, Ye L, Xu G, Ma Z, Cao H, Zhang Y, et al. Quantitative and qualitative analysis of *Artemisiae verlotori* Folium and *Artemisiae argyi* Folium by high-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry. J Sep Sci 2023:46:2300063.
21. Chindarkar NS, Park HD, Stone JA, Fitzgerald RL. Comparison of different time of flight-mass spectrometry modes for small molecule quantitative analysis. J Anal Toxicol 2015;39(9):675–85.
22. Lee JH, Seo MK, Ham HJ, Seo S, Shin D, Kim HI. Simultaneous quantitation of 17 female sex hormones in illegal products by validated liquid chromatography-high resolution mass spectrometry and liquid chromatography-tandem mass spectrometry methods. J Sep Sci 2023;2200963.
23. Zhu X, Chen Y, Subramanian R. Comparison of information-dependent acquisition, SWATH, and MS^{all} techniques in metabolite identification study employing ultrahigh-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. Anal Chem 2014;86(2):1202–9.
24. Kumar S, Singh A, Bajpai V, Srivastava M, Singh BP, Kumar B. Structural characterization of monoterpene indole alkaloids in ethanolic extracts of *Rauwolfia* species by liquid chromatography with quadrupole time-of-flight mass spectrometry. J Pharm Anal 2016;6(6):363–73.
25. Kumar S, Bajpai V, Singh A, Kumar B. Identification, characterization and distribution of terpene indole alkaloids in ethanolic extracts of *Catharanthus roseus* using high-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry and the study of their geographical variation. Rapid Commun Mass Spectrom 2018;32(4):319–32.
26. Li W, Xu X, Tang Z, Guo Y, Fei D, Yan N, et al. Analysis of 14 terpenoids and sterols and variety discrimination of *Codonopsis radix* using ultra-high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. J Sep Sci 2023;46(8):2200835.
27. Li D, Cheng X, Jiang Z, Shi Z, Qu C, Yan H, et al. Chemical profiling and quantification analysis of flavonoids in different varieties of Euryales Semen by ultra-high-performance liquid chromatography with tandem mass spectrometry. J Sep Sci 2023;46(7):2200913.
28. Abe F, Chen RF, Yamauchi T, Marubayashi N, Ueda I. Alschomine and isoalschomine, new alkaloids from the leaves of *Alstonia scholaris*. Chem Pharm Bull 1989;37(4):887–90.
29. Pan ZQ, Qin XJ, Liu YP, Wu T, Luo XD, Xie CF, et al. Indole alkaloids from *Alstonia scholaris*: structural evaluation and bioinspired synthesis of alstoscholarisine H. Org Lett 2016;18(4):654–7.
30. Zhao YL, Su M, Shang JH, Wang X, Bao GL, Ma J, et al. Genotoxicity and safety pharmacology studies of indole alkaloids extract from leaves of *Alstonia scholaris* (L.) R. Br. Nat. Prod. Bioprospect. 2020;10(3):119–29.
31. Zhao YL, Yang ZF, Wu BF, Shang JH, Liu YP, Wang XH, et al. Indole alkaloids from leaves of *Alstonia scholaris* (L.)

R. Br. protect against emphysema in mice. J Ethnopharmacol 2020;259:112949.
32. http://academy.gmpcompliance.org/guidemgr/files/Q2(R1) PDF Accessed March 30, 2023
33. https://www.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/SLV_Guidelines_Dietary_Supplements.pdf Accessed March 30, 2023
34. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf Accessed March 30, 2023
35. https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf Accessed March 30, 2023
36. Tena MT, Martinez-Moral MP, Cardozo PW. Determination of caffeoylquinic acids in feed and related products by focused ultrasound solid-liquid extraction and ultra-high performance liquid chromatography-mass spectrometry. J Chromatogr A. 2015;1400:1–9.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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