Enrichment and analysis of quaternary alkaloids from *Zanthoxylum simulans* using weak cation exchange solid-phase extraction coupled with LC-MS

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ABSTRACT

Introduction: Quaternary alkaloids (QAs) are the major alkaloids in several traditional Chinese medicines, especially in Zanthoxylum simulans (Z. simulans). However, few studies on enrichment of QAs from Z. simulans were conducted due to their high polarity and low content.

Objective: To develop a weak cation exchange solid-phase extraction coupled with LC-MS method to enrich and identify QAs from Z. simulans. Meanwhile, the qualitative and quantitative analyses of QAs were carried out based on the optimum conditions of the method.

Methods: Fresh stem bark of Z. simulans was extracted with 70% aqueous methanol and enriched by weak cation exchange (WCX) solid-phase extraction (SPE). A high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) with an electrospray ionisation (ESI) source was used for the qualitative and quantitative analyses of QAs.

Results: Significant improvements were obseved in resolution and abundance of the peaks with WCX-SPE. The linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined for this analytical method. The linear relationship (A = 338.85C-187.72, R2 = 0.99) was explored in the range of 0.5-312.5 μg/mL for chelerythrine. The LOD and LOQ for

chelerythrine standard solutions were 0.0539 μg/mL and 0.1798 μg/mL, respectively. In addition, twenty two peaks were detected successfully with WCX-SPE and nine of them are undetectable without the processing of WCX-SPE.

Conclusion: A highly selective and efficient method for simultaneous enrichment and identification of QAs from crude extract of Z. simulans was developed for the first time by combining WCX-SPE with LC-MS.

KEYWORDS

Zanthoxylum simulans, quaternary alkaloids, weak cation exchange, solid-phase extraction

1 | INTRODUCTION

The genus *Zanthoxylum* (Rutaceae) consists of about 250 species of deciduous shrubs, and 39 species with 14 varieties have been found in China.[1] *Zanthoxylum simulans* (*Z. simulans*), a common prickly shrub, has the effects of relieving swelling and pain, detoxification, and diminishing inflammation.[2] Previous phytochemical studies on this plant have led to the identification of various compounds，such as volatile oils,[3] alkaloids, coumarins, lignans, terpenoids, and sterols.[4,5] Among them, lignans and neolignans mainly distribute in the stem wood when compared with any other parts of this plant.[4] Alkaloids, mainly found in the root or the stem bark [6,7] of *Z. simulans* have been of immense interest due to their bioactivities,[8-10] and quaternary alkaloids (QAs) as major alkaloids in this plant possess significant antitumour activities. For instance, nitidine displayed inhibitory activity on hepatic carcinoma cells by inhibiting the JAK1/STAT3 signal pathway *in vitro*,[11] chelerythridine and sanguinarine showed dose-dependent inhibitory activity by damaging the DNA of leukaemia carcinoma cells (L1210) *in vitro*,[12] and fagaronine acted as angiogenesis inhibitors on leukaemia cancer cells.[13] To explore the antitumour activities and the subsequent mechanisms of action regarding QAs from *Z. simulans*, one of the most important steps is to enrich and identify them from complex crude plant extracts.

In this context, we set out to develop and optimize the method for the simultaneous enrichment and analysis of alkaloids of interest. Alkaloids, as an important subgroup of plant secondary metabolites, are a type of nitrogen-containing organic compounds, and most of which have complex nitrogen heterocyclic structures. Meanwhile, alkaloids often co-exist with a large number

of other compounds, and are in extremely low content. Most types of alkaloids are easy to be dissolved in organic solvents rather than in water, while quaternary alkaloids can be dissolved both in water and alcohol. QAs, as soluble alkaloids, cannot be extracted directly from aqueous solution by organic solvents due to their specific polarity with very low contents in the plant, thus the pre-concentration of QAs from crude plant extract is extremely difficult. Thus it is urgent to develop an efficient method to enrich QAs. Therefore, effective enrichment of QAs from *Z. simulans* is the first important step prior to their qualitative and quantitative analyses.[14] In the previous study, repeated column chromatography and further purification combined with nuclear magnetic resonance (NMR) techniques were commonly employed to isolate and identify pure QAs from *Z. simulans*.[15] Although these methods could offer efficient and precise results, it was relatively time-consuming, labour-intensive, and expensive for precious samples. In an effort to improve the extraction efficiency as much as possible, Reinecke salt based colorimetric method was reported.[16] Although precipitation, filtration and further purification were seemed to be effective in this method, the cations, such as $Cr4^{+}$ and Ag^{+}, are not environment-friendly, and also the labile factors. For instance, the selected reagents to precipitate, cation varieties, and time to precipitate could cause troubles leading to the failure to enrich alkaloids of interest. In addition, with the fast development of separation and analytical technologies, some instruments with high separation efficiency and sensitivity have been applied to the alkaloids analysis from the *Zanthoxylum* genus, including but not limited to high-speed counter current chromatography (HSCCC), capillary electrophoresis (CE), reversed phase high-performance liquid chromatography (RP-HPLC), and high performance liquid chromatography combined with electrospray tandem mass spectrometry and nuclear magnetic resonance (LC-ESI/MS/NMR).[17-21] However, the aforementioned instruments could not improve the analytical results significantly without proper pre-purification due to the complexity and high polarity of QAs. Therefore, we aimed to develop a cost-effective and environment-friendly method to enrich and purify QAs from *Z. simulans*.

Currently, some researches and applications of new technologies have greatly improved the efficiency of enrichment, meanwhile saved energy and time. For instance, macroporous resin is a kind of organic polymer adsorbents, which has frequently been reported in the enrichment and purification of alkaloids because of its good efficiency,[22] but the method has some limitations

because the efficiency is restricted by many factors such as pH. To overcome the above limitations, ion exchange resin has demonstrated the advantages of low cost, high efficiency, good maneuverability, and low pollution, which has been widely used in the enrichment and purification of acid and alkaline components in natural products.[23, 24] Fortunately, WCX resin, with anions such as $RCOO^-$, can be unexceptionably combined with QAs in the aqueous solution, [25] thus, successful enrichment of QAs can be achieved by using this strategy. With the carboxylic acid functional groups, WCX could combine with positive ion in water in the form of negatively charged groups because of the dissociation and cation exchange. It is difficult to dissociate in accompany with cation exchange in low pH and prefers to alkaline, neutral, and slightly acidic solutions. At this point, the WCX material, with the advantage of easy to be regenerated, might be applied to enrich and obtain higher content of QAs. WCX chromatography has played an important role in the field of separation and purification of samples, such as drug, urine and plasma samples.[21, 26-30] With WCX, the effectiveness of separation and purification was markedly improved. However, WCX has not been reported to enrich and purify QAs from *Z. simulans*. Taken all the points above into consideration, the application conditions of WCX were optimized and applied to the enrichment and analysis of QAs from *Z. simulans* in this study. As a result, a simple WCX based method for the simultaneous enrichment and analysis of QAs was firstly developed and proved to be an efficient method for fast analysis and quality control of QAs from plants.

2 | EXPERIMENTAL

2.1 | Materials, chemicals and reagents

Fresh stem bark of *Z. simulans* was collected from Wuhan Botanical Garden of the Chinese Academy Sciences (Wuhan, China) in April 2015. After drying below at 30 ^oC, the stem bark materials were crushed and stored in drying apparatus before use.

The reference standards, including magnoflorine, laurifoline, magnocurarine, fagaronine, chelerythrine, were purchased from the national standard substance center and stored at room temperature. Acetonitrile of HPLC grade was purchased from Thermo Fischer Scientific Inc. (USA). Other organic solvents were of analytical grade and purchased from Sino-pharm Chemical Reagent Co. Ltd. (Shanghai, China). Three standards, nuciferine, lycorine and bhelerythrine, were

obtained from Tauto Bio-tech Co. Ltd. (Shanghai, China). Water for LC and LC-MS were prepared from EPED (Nanjing Yeap Esselte Technology Development Co., Ltd. Nanjing, China). Mix-mode Weak Cation Exchange Solid Phase Extraction columns (WCX) were bought from ANPEL Scientific Instrument (Shanghai, China) Co., Ltd.

2.2 | Instruments and conditions

The flow rate of SPE was 1 mL/min and the samples drying were recorded on nitrogen drier (Organomation N-EV AP) with 45 °C and 12 L/min. The analysis was performed on a Thermo Accela 1250 HPLC (Thermo Fisher Scientific, USA) combined with an auto-sampler and a VWD detector. A 10 μL aliquot of sample solution was injected and separated on a Phenomenex ODS (2) column (5 μm, 2 mm × 150 mm) at 25 °C. The chromatograms were observed at a wavelength of 280 nm, and the flow rate was set at 0.2 mL/min. Mobile phase A and B were 5 mM ammonium acetate solution, and acetonitrile, respectively. The gradient was set as follows: 0-5 min, 5% (B); 5-50 min, 5-60% (B). For ESI-MS/MS analysis, a Thermo Accela 600 LC system with a VWD detector and a TSQ Quantum Access MAX mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) were applied to detect alkaloids in the positive ion mode. The MS conditions were optimized as follows: mass range from 200-1000 Da; spray Voltage, 3.0 kV; capillary temperature, 250 °C; sheath gas pressure, 40 psi; aux gas pressure, 10 psi.

2.3 | WCX extraction procedures and sample preparation

Immersing in 70% aqueous methanol for 12 h, crushed stem bark of *Z. simulan*s was extracted ultrasonically for 30 min in triplicates. After centrifugation, the supernatants were combined and condensed to afford residues, which were then dispersed in methanol (equal to 1 g plant materials in 1 mL methanol). The WCX Solid Phase Extraction conditions were optimized and used to enrich crude QAs from *Z. simulans* as follows: 1) WCX cartridges were preconditioned with methanol (3 mL) and 10% methanol (3 mL); 2) An aliquot of 200 μL crude methanol extract of *Z. simulans* was diluted in 10% methanol and further dispersed to 10 mL for three times. After centrifuged at 10000 rpm for 5 min，the samples were loaded to the preconditioned cartridges; 3) Water (1 mL) and methanol (5 mL) were used to wash out the unbounded components; 4) Crude QAs were eluted by methanol with 5% formic acid (5 mL). Then, the final eluents were collected

and dried by nitrogen drier, and the residues were dissolved with 10% methanol and filtered with 0.22 μm micro-filter membrane before the LC-MS analysis.

2.4 | Method validation

To validate the analysis method for the determination of QAs, a series of experiments were carried out. The regression equation and correlation coefficient were determined by integrating peak area of different concentrations of standard solutions accompany with a linear regression analysis. To assess the precision and accuracy of the method by calculating the relative standard deviation (RSD) and the recovery rate of inter-day, reference solution of chelerythrine with three different concentrations (0.94 μg/g, 0.63 μg/g, 0.31 μg/g) was added to the sample of *Z. simulans*, then the sample was extracted though WCX solid-phase extraction column and injected for HPLC analysis. For intra-day stability test, the chelerythrine standard solution was analyzed 5 times within one day (n = 5), the results were measured by relative standard deviations (RSD). The recovery of method was evaluated by adding spiked samples. The limit of detection (LOD) and limit of quantification (LOQ) for chelerythrine standard solutions were calculated based on signal-to-noise ratio (S/N) of 3 and 10, respectively.

3 | RESULTS AND DISCUSSION

3.1. Effectiveness of WCX SPE on the enrichment of quaternary alkaloids (QAs)

As expected, crude extracts of *Z. simulans* were then processed by WCX-SPE, and subjected to HPLC analysis. Fig.1 shows the chromatograms in the HPLC (280 nm) analysis of crude extracts without (A) and with (B) WCX-SPE. It was observed that the peak shape of the sample with WCX-SPE was remarkably better than that without WCX-SPE. Meanwhile, the resolutions of most peaks observed were significantly improved, and 22 peaks were clearly resolved in Fig.1B, which clearly indicated that WCX-SPE can greatly enhance the enrichment and analysis of QAs from *Z. simulans*.

3.2 | Optimization of WCX procedures

In order to achieve the best enrichment efficiency for QAs from plant extracts, some relevant key parameters, including pH of samples, desorption reagents, specificity, and amount of rinse

reagents, sample sizes were systematically investigated. For instance, proper acidic samples might be good for QAs to dissolve, but improper acidic levels could cause low recoveries of QAs. As for alkalized samples, significant higher pH might lead to poor extraction efficiencies for QAs because of the competitive binding to carboxyl group between QAs and cation in solution. Thus, the effects of pH on the enrichment efficiency were evaluated in the range of 3.0-11.0 to obtain the best binding capacity. As shown in Fig. 2A, after subjected to WCX, the highest recovery for QAs was achieved at around pH 7.0. Thus, pH 7.0 was selected in the following studies. Based on another application by Qiu *et al.*,[30] 5 mL of desorption reagents were used to elute QAs from WCX in the following experiments. To select the suitable desorption reagents to improve the efficiency, the most commonly used desorption reagents, such as 5% formic acid in methanol, 5% formic acid in acetonitrile, and 5% formic acid in acetone were investigated.[26, 29, 35] Fig. 2B shows that 5% formic acid in methanol was the best with 96.0% recovery rate of chelerythrine, which was eventually selected as the desorption reagents for the complex samples from *Z. simulans*. To evaluate the specificity of WCX to QAs, the recovery of two pure compounds, chelerythrine and nuciferine, were compared, since distinctive polarity discrepancy of compounds could make a great contribution to the specificity of cation exchange solid-phase extraction.[29]

In general, a variety of alkaloids, like different types of tertiary alkaloids and quaternary alkaloids from *Z. simulans*, could possess different polarities. Most of the alkalogenic compounds present alkalinity in 10% methanol solution, and the alkalinity of the type of tertiary alkaloids is slightly weaker than that of QAs. Accordingly, the mixed standard solution, containing nuciferine (tertiary alkaloid) and chelerythrine (quaternary alkaloid), was extracted by WCX solid-phase extraction column, in order to investigate the special properties of the mixed weak cationic solid phase extraction column to quaternary alkaloid. Based on evidences above, WCX-SPE had obvious enrichment for chelerythrine (quaternary alkaloid) and no obvious enrichment effect to nuciferine (tertiary alkaloid). To investigate the volumes of the rinse reagents for WCX-SPE, the mixed standard solution with lycorine (tertiary alkaloid) and chelerythrine (QA), was extracted by WCX-SPE, and then eluted with 3 mL, 5 mL and 7 mL methanol, respectively. Finally, the optimal volume of the rinse reagent (methanol) was confirmed by calculating the recovery rate of the two standard alkaloids. As shown in Fig. 2C, the recovery rate of chelerythrine slightly increased with the volume of methanol from 3 mL to 5 mL, while the recovery rate of lycorine

decreased significantly in the meantime. The recovery rates of lycorine and chelerythrine showed no significant change, with the volumes of methanol increase from 5 mL to 7 mL. Thus, the volume of rinse reagent (methanol) was defined as 5 mL in this study, in order to reduce the non-QAs content in the sample with WCX.

Since the mixed mode weak cationic solid-phase extraction column used in this study was packed with 500 mg of material in a 3 mL column, the ion exchange capacity of each column was limited. In this way, appropriate sample size was extremely vital for the successful enrichment of QAs, and underloading or overloading the sample would cause some alkaloids from the *Z. simulans* undetectable or with poor resolution, and even damage the column. Chelerythrine, a representative type of QAs, while not presented in *Z. simulans*, was then selected to optimize the loading amount of samples. To get the recovery rate of the chelerythrine, 0.2 g and 0.4 g samples of *Z. simulans* were prepared with the addition of certain amount of reference substance chelerythrine, respectively. Then, the samples were subjected to WCX solid-phase extraction column. The recovery rates of added chelerythrine were 93.9% and 106.0%, respectively, when the samples loaded are 0.2 g and 0.4 g. By comparison of the chromatography analyzed under 280 nm, same peaks were detected for 0.2 g and 0.4 g crude material. Therefore, the sample amount for the analysis of quaternary alkaloid in final could be economically set as 0.2 g.

3.3 | Validation of the proposed method

Table 1 shows the results of linear ranges (LR), limits of detection (LOD), limits of quantitation (LOQ), and relative standard deviations (RSD) for a representative QA (chelerythrine). The linearity (A = 338.85C-187.72; where A is absorbance, and C refers to the concentration of chelerythrine) was good with a correlation coefficient (R^2) greater than 0.9999, when the concentrations of chelerythrine ranged from 0.5 μg/mL to 312.5 μg/mL. LOD and LOQ for chelerythrine were 0.0539 μg/mL and 0.1798 μg/mL, respectively. To assess the precision and accuracy of the method by calculating the RSD and the recovery rate of inter-day, the reference solutions of chelerythrine with three different concentrations (0.94 μg/g, 0.63 μg/g, and 0.31 μg/g) was added to the sample of *Z. simulans*, then the sample was subjected to WCX solid-phase extraction column prior to HPLC analysis. For intra-day stability test, the chelerythrine standard solution was analyzed 5 times within one day (n = 5), and the RSD was 0.67%, which indicated

good stability of the method. The recovery rates (RR) of three difference concentrations of chelerythrine were 107.8%, 105.8%, and 93.4%, respectively; and the average recovery rate (ARR) was 102.3%. The results clearly indicated that the proposed method had good accuracy, and could be used to detect the content of other QAs.

3.4 | Applications of WCX-SPE for the enrichment and fingerprinting analysis of quaternary alkaloids

Under the optimized conditions, 0.2 g of *Z. simulans* was used, and the total QAs of *Z. simulans* were enriched by WCX, and eluted with 5% formic acid in methanol solution, and then subjected to HPLC and LC-MS in order to further confirm the selectivity of WCX to QAs, and identify quaternary alkaloids in *Z. simulans.*

3.4.1 | Fingerprinting analysis of total QA in *Z. simulans*

Chelerythrine, the crude extract of *Z. simulans*, and total quaternary alkaloids enriched from *Z. simulans* by WCX were eluted under the chromatographic conditions shown in section 2.2, the chromatograms of three samples were presented in Fig. 3 (0-40 min). With the application of the WCX-SPE, QAs from the crude extract of *Z. simulans* were successfully enriched and a majority of non-QAs were removed because of the high selectivity of WCX for quaternary amines. There were 22 compounds detected under 280 nm, and peak 18 was identified according to the retention time of chelerythrine and the corresponding fragmentation patterns in LC-MS/MS.

3.4.2 | Qualitative and quantitative analysis of QAs in *Z. simulans*

The samples with WCX-SPE were analyzed by LC coupled with a TSQ Quantum Access MAX mass spectrometer system, and the LC chromatogram is presented in Fig. 3. The information of detected compounds is tabulated in Table 2, which lists contents, retention times (t_R) and MS fragment ions. Based on MS spectra of detected peaks, 15 alkaloids exhibited their quasi-molecular ions $[M]^+$ or $[M+H]^+$, and were identified from *Z. simulans* and shown in Fig. 4, which included four benzophenidine alkaloids (fagaronine 3-glucoside, fagaronine, 8-*O*-demethylchelerythrine, chelerythrine), three *N*-methyl-tetrahydrocorberine alkaloids (*N*-methyltetrahydrocolumbamine, *N*-methylcanadine or its isomer), three aporphine alkaloids

(magnoflorine, laurifoline, 10-demethyl-magnoflorine), one protoberberine alkoloid (palmatine), and four benzylisoquinoline alkaloids (magnocurarine or its isomers, 8-methoxy-isotembetatrine, and isotembetarine). Their MS/MS data and fragmentation patterns were in good agreement with those reference compounds or literatures. In more details, the interpretations of MS/MS spectra for different types of alkaloids would be discussed by taking some representative alkaloids as examples. For benzophenidine type of alkaloids, peak 18 with the parent ion at *m/z* 348 was further discussed; the product ions at *m/z* 332, 318, 304 and 290 indicated different neutral losses of 16, 30, 44 and 58 Da, which were corresponding to CH_4, CH_2O, C_2H_4O and C_2H_5CHNH, respectively. Based on the fragment ions and the analysis above as well as the data in the literature, peak 18 could be proposed as chelerythrine.[36] As shown in the MS/MS spectrum, *m/z* 190 and 165 were the most intensive abundance fragments, indicating the RDA cleavage of the mother ion. The further losing of $\cdot CH_3$ from *m/z* 190 produce the fragment at *m/z* 175. Based on the reported literature, peak 14/16 with the molecular ion ($[M + H]^+$ at *m/z* 354 were tentatively identified as *N*-methylcanadine or its isomer.[37] In the MS/MS spectrum of peak 3 and 8, same molecular ion $[M+H]^+$ at *m/z* 342 and common fragment ions $[M-(CH_3)_2NH]^+$ at *m/z* 297 and $[M-(CH_3)_2NH-CH_3]^+$ at *m/z* 282 were observed because of the cleavage of atom adjacent to N atom. Due to the drop of CH_3OH from *m/z* at 297 led to $[M-(CH_3)2NH-CH_3OH]^+$ at *m/z* 265. The distinguishing fragment *m/z* at 237 was observed in the MS/MS spectrum of compound. Based on the reported literature, peak 3 and 8 were identified as magnoflorine and laurifoline,[38] respectively. Peak 19 had $[M+H]^+$ at *m/z* 352. The MS/MS fragments of peak 19 at *m/z* 337, 334, 322, 320, 308 and 294 were consistent with palmatine reported previously. As a result, peak 19 was definitely identified as palmatine.[39] For peak 4 and 9, the characteristic and intensive fragment at *m/z* 209 and 107 were observed due to the β cleavage of $[M+H]^+$ at *m/z* 314. The further cleavage of bond adjacent to *N* atom from *m/z* 209 led to fragments at *m/z* 194 and 166. Due to different retention time at 19.91 and 24.54 min but same MS/MS fragments, peak 4 and 9 with same molecular ion $[M + H]^+$ at 314 *m/z* were identified as magnocurarine or its isomers.[40] As shown in the Fig.3, the contents of QAs were determined using the method with external standard, and the contents of magnoflorine (peak 3), laurifoline (peak 8), magnocurarine or its isomer (peak 9), fagaronine (peak 15) and chelerythrine (peak 18) are higher than the other alkaloids, which are 234.2 μg/g, 68.8 μg/g, 87.4 μg/g, 371.7 μg/g and 193.0 μg/g, respectively. Meanwhile, the contents of these

five compounds account for more than 5% of the detected alkaloids in *Z. simulans*. In addition, nine alkaloids were detected from the sample enriched by WCX-SPE, but were not detected without enrichment.

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Analytical	LR (µg/mL)	R^2	LOD (µg/mL)	LOQ (µg/mL)	RSD (%) / Intra-day	ARR (%)
Chelerythrine	0.5-312.5	0.9999	0.0539	0.1798	0.67	102.3

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Table 2 Alkaloids detected in *Z. simulans* by LC-MS/MS.

Peak NO.	Rt / min	M^+/ $[M+H]^+$	MS/MS spectrum	Identification	Contents (μg/g)	Relative contents (%)
1	4.50	314	314, 213, 140, 121, 103, 97	Unidentified*	35.9	2.98
2	10.18	222	222, 207, 191, 179, 164, 58	Unidentified*	3.0	0.25
3	18.10	342	342, 297, 282, 265, 247, 237, 219, 207, 191	Magnoflorine	234.2	19.46
4	19.91	314	314, 269, 237, 219, 209, 194, 192, 166, 137, 119, 115, 107	Magnocurarine or its isomer	9.4	0.78
5	21.60	358	358,313,298,283,267,206,189,174,163,158,151,137	8-Methoxy-isotembetatrine	8.7	0.73
6	22.28	344	344, 314, 301, 269, 239, 207, 175, 143, 137	Isotembetarine	9.5	0.79
7	22.90	328	328, 313, 283, 268, 189, 151, 121, 107	Unidentified	4.8	0.40
8	23.51	342	342, 297, 282, 265, 250, 233, 222, 205	Laurifoline	68.8	5.72
9	24.54	314	314, 269, 237, 219, 209, 194, 192, 166, 137, 119, 115, 107	Magnocurarine or its isomer	87.4	7.26
10	25.75	512	512, 350, 335, 307	Fagaronine 3-glucoside	21.7	1.80
11	26.26	328	328, 283, 251, 223, 208, 175, 143, 121	10-Demethyl-magnoflorine	2.5	0.20
12	27.61	454	454, 340, 322, 226, 209, 114, 96	Unidentified*	5.8	0.48
13	28.31	356	356, 192, 177, 149	*N*-Methyltetrahydrocolumbamine	1.9	0.16
14	28.82	354	354, 190, 175, 165	*N*-Methylcanadine or its isomer*	4.4	0.36
15	30.58	350	350, 335, 320, 307, 292, 264	Fagaronine*	371.7	30.88
16	32.21	354	354,190,175,165	*N*-Methylcanadine or its isomer	43.0	3.57
17	33.57	334	334, 319, 304, 291, 276, 262	8-*O*-Demethylchelerythrine	2.5	0.21
18	33.98	348	348, 332, 318, 304, 290	Chelerythrine	193.0	16.04
19	38.48	352	337, 334, 322, 320, 308, 294	Palmatine*	21.5	1.79
20	39.19	378	378, 363, 334, 319	Unidentified*	19.2	1.59
21	39.97	274	274, 256, 106, 88	Unidentified*	22.76	1.89
22	41.29	594	594, 533, 385, 348, 193, 149	Unidentified*	8.0	0.67

*compounds were not detected before WCX-SPE, but were successfully detected after WCX-SPE

Figure captions:

FIGURE 1 The chromatograms of total extracts of *Z. simulans* without (A) and with (B) WCX. Twenty two peaks were detected with WCX-SPE and nine of them are undetectable in absence of WCX-SPE.

FIGURE 2 Effects of pH of sample loading solvents (A), desorption reagents (B), and volumes of rinse reagents (C) on the extraction efficiency. A): The highest recovery for QAs was achieved at around pH 7.0 after subjected to WCX; B): 5% formic acid in methanol was eventually selected as the desorption reagents; C): The volume of rinse reagent (methanol) was defined as 5 mL.

FIGURE 3 The chromatograms of chelerythrine (a), crude extract of *Z. simulans* (b) and total QAs enriched from *Z. simulans* by WCX (c). The sample with WCX-SPE has twenty-two QAs in c, while the resolution and abundance of the peaks in b is not so satisfactory without WCX.

FIGURE 4 The structures of compounds detected from *Z. simulans*.