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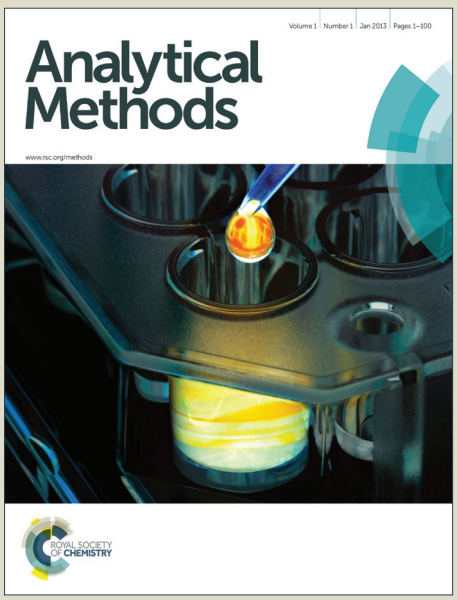

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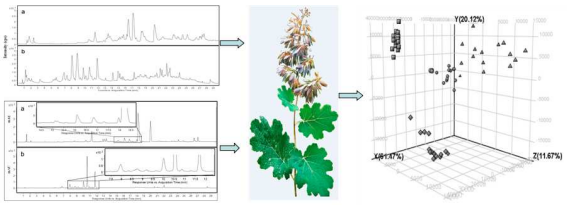

An improved separation method for classification of *Macleaya cordata* from different geographical origins

Zhi-Xing Qing[1,2 †], Xiu-Bin Liu[1†], Hui-Min Wu[2], Pi Cheng[1], Yi-Song Liu[1], Jian-Guo Zeng[1,2,3*]

1. National Herbal Medicine Production Technology Center (Hunan), Hunan Agricultural University, Changsha 410128, China;

2. Hunan Co-Innovation Center for Utilization of Botanicals Functional Ingredients, Hunan University of Chinese Medicine, Changsha 410208, China;

3. Hunan Engineering Research Center of Botanical Extraction, Changsha 410331, china

*Correspondence to: *Jian-Guo Zeng, College of horticulture and landscape, Hunan Agricultural University, Changsha 410128, China.* E-mail: *ginkgo_1world@163.com*

†: *These authors contribution equally to this work.*

Keywords: polar compounds, alkaloid, *Macleaya cordata,* Mass spectrometry

Abstract:

In this study, an improved separation method for metabolomic study of polar and basic secondary metabolites by high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (HPLC-Q-TOF-MS) using an "Xcharge C18" column has been developed. Good retention for polar compound and perfect peak shape for basic components was achieved. Classification four different major origins of *Macleaya cordata*, which is a traditional folk medicine mainly used as a popular natural feed additive (*e.g.* Sangrovit®) in Europe and Asia since 2002, was performed by principle component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA).

PCA of analysis data showed a clear separation among four different geographical origins. The biomarkers such as *N*-methyl-7-demethoxylhydrocotarnine and 13-methylcryptopine accountable for variation were tentatively identified by their tandem mass spectrometry (MS/MS) fragmentation behaviors. The proposed analytical method was shown to be a useful tool for the metabolomic study of polar compounds and alkaloids containing plants.

1. Introduction

Qualitative and quantitative analysis of the metabolites, mainly including primary metabolites (*e.g.* carbohydrates, lipids and amino acids) and secondary metabolites (*e.g.* alkaloids, terpenoids and flavonoids), in biological samples has been paid more and more attention in recent years.[1] However, the unbiased and simultaneous determination of the secondary metabolites is far from easy. Thousands of secondary metabolites existed in tissue and biofluid are the main challenge for metabolic analysis. Due to their different physico-chemical properties and abundance. They range from hydrophilic to hydrophobic, acidic to basic molecules at picomolar to millimolar concentrations.[2] Based on this fact, separation is an essential part of the metabolic study, strongly affecting on both resolution and quantification.[3] Chromatography methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), capillary high-performance liquid chromatography (capHPLC), capillary electrophoresis and capillary electrochromatography, have been used on-line and off-line with mass spectrometry for the separation, identification and quantitation of secondary metabolites from different samples.[2,4] Among the MS-based method, HPLC-MS is increasingly used for metabolic studies due to HPLC can separate

different classes of compounds though different chromatographic modes and MS has the ability to determine the secondary metabolites at pg/ml range. Reversed phase liquid chromatography (RPLC) is the most popular HPLC mode used in the metabolomic research for its universality, high separation efficiency and good reproducibility. [4-6]

However, it is still a great challenge to separate polar and basic secondary metabolites for conventional RPLC. Polar secondary metabolites are important components in the cellular regulatory process, and their changes in quality or quantity demonstrate that biosystem was motivated to some extent. Owing to the limitation of separation, hardly any studies focused on the profiling of polar secondary metabolites until hydrophilic interaction chromatography (HILIC) was proposed by Alpert.[7] Basic secondary metabolites are widely distributed in metabolic samples, especially in higher plants whose main ingredient is alkaloid, such as *Papaver somniferum L.*, *Rhizome coptidis*, *Macleaya cordata* and *Corydalis rhizome*. Severe peak tailing often takes place using conventional RPLC for alkaloids, even when a very small amount is injected.[8] It is contributed to the strong ionic interactions between basic alkaloids and the acidic residual silanols on the surface of silica matrix.[9] a new type of polar modified reversed phases liquid chromatography stationary phase was developed by a so-called "polar-copolymerized" approach with a commercial name "XCharge C18".[10] It exhibited not only enhanced retention and resolution ability for the polar compounds,[11] but also excellent peak shape for basic compounds without any salt buffer or ion-pair reagents.[10,12]

In this work, an improved separation method, mainly focused on the simultaneous separation of polar and basic compounds for metabolomic study of alkaloids containing plant was developed. Classification of *M. cordata* from different major geographical

origins was used to test this method. This medical plant is rich in quaternary isoquinoline alkaloids, which were used to inhibit bacterial as a human medicine for external use,[13] promote animal growth as a veterinary medicine,[14] protect crop as a pesticide.[15] Finally, polar and basic compounds were simultaneously separated and detected in this study. Their major difference was shown by principal component analysis (PCA) and partial least square discriminate analysis (PLS-DA). Potentially variable compounds were tentatively identified by tandem mass spectrometry (MS/MS) for characterisation of the *M. cordata* from four geographical origins.

2. Experimental

2.1. Chemicals

Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany), formic acid with a purity of 99% was purchased from ROE (Newark, New Castle, USA), and deionized water was produced by a Milli-Q purification system (MA, USA).

2.2. Materials and Sample preparation

A total of 60 *M. cordata* plant's fruits were collected from four different major origins: Jinzhai (Anhui province), Qimen (Anhui province), Changsha (Hunan province) and Loudi (Hunan province) in August, 2013 and were authenticated by Prof Jian-Guo Zeng (Hunan Agricultural University, China). 0.5 g fine powder (60 meshes) of each sample was added into 100 ml 50% aqueous methanol solution, ultrasonically extracted at 100 Hz for 60 min, and then cooled at the room temperature. After compensating for the lost weight of methanol, the extracted solution was centrifuged at 13,000 rpm (8493g) for 10 min. The supernatant was filtered through a 0.22 μm nylon filter before HPLC analysis.

2.3. HPLC-Q-TOF/MS

An Agilent 1290 LC system (consisting of vacuum degasser, autosampler, rapid resolution binary pump and thermostatted column compartment) coupled with a 6530 Q-TOF/MS accurate-mass spectrometry (Agilent Technologies, USA) was used for HPLC-Q-TOF/MS analysis. The two chromatographic columns used in this study were XCharge C18 (150 mm × 2.1 mm, 5 μm, Acchrom Co. Ltd, China) and Zorbax Eclipse Plus C18 (150 mm × 4.6 mm, 5 μm, Agilent Technologies, USA). The aqueous constituent of the mobile phase (eluent A) differed depending on ionization polarity setting: 0.2% formic acid aqueous solution and aqueous solution were used in positive and negative modes, respectively. The organic modifier of the mobile phase was 0.2% formic acid in acetonitrile (eluent B). A linear gradient was optimized as follows, 0-2 min, 0% B; 2-5 min, 0-10% B; 5-6 min, 10-11% B; 6-16 min, 11-35% B; 16-26 min, 35-100% B; 26-27 min, 100% B; flow rate was set at 0.35 and 1 mL/min for those two columns, respetively. The injection volume was 5 μL and the column temperature was maintained at 30 ^{o}C in each run.

The mass spectrometer was operated either in positive electrospray ionization (ESI^{+}) or negative electrospray ionization (ESI^{-}) mode, parameter settings used for the measurement were as follows: capillary voltage: 4kV (positive ion mode) and 3.5 kV (negative ion mode); Nozzle voltage: 0kV (positive ion mode) and 1 kV (negative ion mode); nebulizer pressure: 50 psi; drying gas: 6 L/min; gas temperature: 300 ^{o}C; skimmer voltage: 65 V; OCT1 RF Vpp: 750 V; fragmentor voltage: 135 V. Data were acquired using the extended dynamic range mode (2 GHz) and collected in the full-scan mode from *m/z* 100 to 1000 in centroid mode. The TOF mass spectrometry was calibrated routinely before sample analysis using reference masses at *m/z* 121.0855, 922.0922

(positive ion mode) and 119.0362, 980.0164 (negative ion mode) to obtain high-accuracy mass measurements. The targeted MS/MS experiments were operated using variable collision energy (10-50 eV), which was optimized for each potential biomarkers.

2.4. Data analysis

The acquired data was firstly analyzed using the molecular feature extraction algorithm of the MassHunter Workatation software (version 3.01, Agilent Technologies, USA). The resulting feature files for each sample were created to the ".cef" files and subsequently exported into the mass profiler professional (MPP) software (version B 02.00, Agilent Technologies, USA) for further processing. After peak finding, alignment, and filtering of raw data, a list of the ion intensities of all compounds with their corresponding retention time and *m/z* was generated. In the next step, this data was analyzed by using PCA and PLS-DA. The marker compounds were tentatively identified by their MS/MS fragmentation behaviors.

3. Results and discussion

3.1. Development of the separation method

Semi-polar and apolar secondary metabolites can be well separated based on their hydrophobility, while polar secondary metabolites typically elute in the solvent front. In this study, the polar-copolymerized C18, named XCharge C18 for commercial use, which was successfully used to separation and purification of polar compounds from *Radix Isatidis*,[11] was employ for the separation of polar secondary metabolites of *M. cordata*. As shown in Fig.1, Visual examination indicated that polar compounds, eluted from 0.5-4min, were well retained and separated on XCharge C18, which was better than on

conventional Zorbax Eclipse Plus C18. The detected ions using XCharge C18 (828 ions) is much more than Zorbax Eclipse Plus C18 (325 ions) (ions whose absolute response in higher than 200 counts in the retention time of 0.5-4min were recorded using MassHunter software).

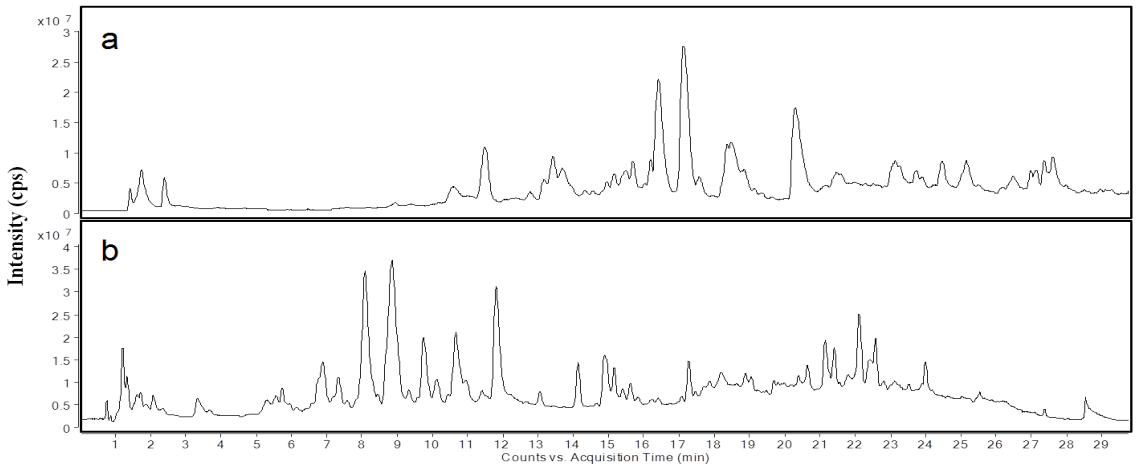

Fig.1 TIC chromatogram on Zorbax Eclipse Plus C18 (a) and XCharge C18 (b).

For metabolomic study of alkaloids containing plants, e.g., *M. cordata*, good peak shape assures the results of detection, data mining and final conclusion. As discussed above, polar-modified C18 not only has the advantage of good retention of polar secondary metabolites, but also improvement of peak shape of basic molecules. In this work, separation efficiency for basic compounds of XCharge C18 is much higher than other commercial well-known C18 in the market. By using the XCharge C18 column, symmetrical peak shape were obtained and shown in Fig.2, while tailing peaks were observed using Zorbax Eclipse Plus C18.

3.2. Optimization of RPLC-MS condition

In the current study, non-targeted metabolomic study of *M. cordata* was performed. In order to obtain profiles containing as many compounds as possible, ultrasonic extraction was employed to deal with the large-amount of *M. cordata* samples due to the good

repeatability and simplicity. In order to extract both the high water solubility and low polarity compounds with high efficiency, 50% aqueous methanol was selected as the extraction solvent.

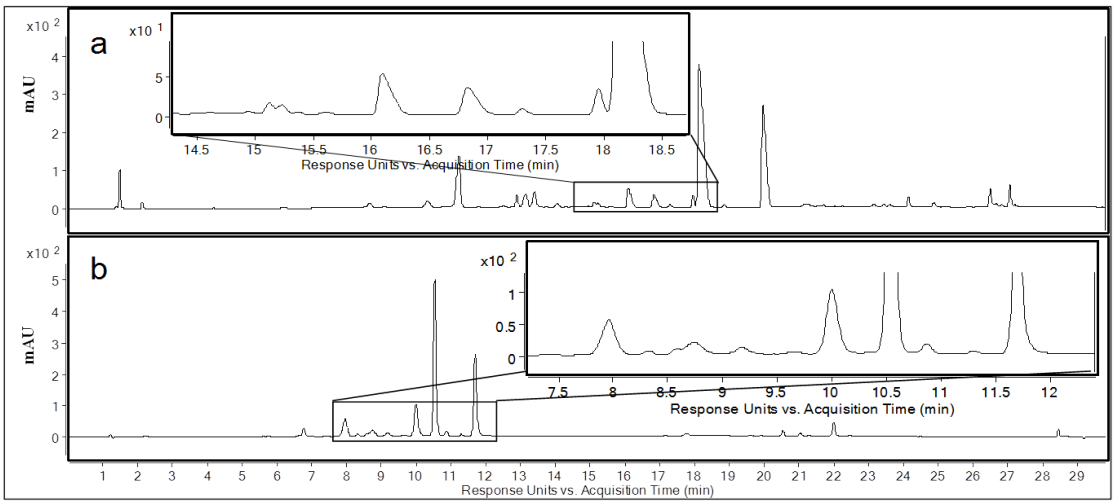

Fig.2 UV chromatogram (280nm) on Zorbax Eclipse Plus C_{18} (a) and XCharge C_{18} (b).

As discussed in the previous study, XCharge C18 is a kind of unique separation material, which can separate alkaloids with excellent peak shape without any salt buffer or ion-pair reagents.[12] Formic acid was chosen as the mobile phase additive, not only assuring the reproducibility of LC separation, but also improving the response in the MS detection owing to alkaloids are prone to be protonated at lower pH. In addition, 100% aqueous solvent was used as the initial mobile phase to enhance the retention of polar metabolites/alkaloids.

Apart from the chromatographic conditions to be defined, the quality control (QC) of the method was also explored here. To confirm the quality control, eight available reference compounds including chelerythrine, sanguinarine, allocryprotopine, protopine, berberine, oxysanguinarine, dihydrosanguinarine, dihydrochelerythrine, which were separated from *M. cordata* in our laboratory, were mixed as the quality control sample to

remain the same instrumental conditions during the whole analytical process. The QC sample was injected every seven samples in the run sequence. The eight representative peaks were chosen to evaluate the deviation of the method of analysis. In all experiments, the retention time variability of the peaks on both columns were determined to be 3 s or with a relative standard deviations (RSD) value less than 6%. The variation *m/z* value of each reference substance was less than 5 ppm and the RSD of the ion intensities were all below 5%. These results revealed the excellent stability and reproducibility of chromatographic separation and MS detection even when 100% aqueous solvent was used as the initial mobile phase in this experiment.

3.3. Molecular feature extraction and data pretreatment

For the metabolomic profiling, multi-dimensional and huge-amounts of LC and MS data were generated, dealing with the LC-MS-based data manually was an extremely difficult task. Therefore, molecular feature extraction (MFE) was employed as an algorithm, enabling automatic extraction of ions corresponding to a certain compound present in samples. During electrospray ionization process, multiple signals, including adducts, dimmers, isotopes and multiply charged species in positive and/or negative mode, which originated from a single compound can be merged into molecular features (MFs) and further handled as a single variable.[16] All the information of compounds would be extracted as MFs, characterized by retention time and accurate mass combined with their abundance. The feature extraction algorithm took all ions into account exceeding 200 counts with a charge state equal to one in the retention time range from 0.5 to 27 min. The abundance was calculated by MassHunter software as the sum of the isotopic and adducts peaks that correspond to a single molecular feature. Peak abundance (the sum of

the isotopic and adduct peaks) values greater than 7,000 in positive mode were reserved. Using this approach, MFs of the secondary metabolites were distinguished and extracted as ".cef" files for the subsequent analysis.

To minimize complex data, mass profiler professional (MPP) software was employed to perform the further filtering procedures. In the first step, chromatographic alignment and normalization of retention time and *m/z* values were carried out using a tolerance window of 0.15 min and 2 mDa, respectively. In the next step, all the MFs that were not present in at least 80% of samples in at least one group were removed. In the third step, MFs were further filtered based on *p*-values calculated by one-way ANOVA. The *p*-value of 0.01 was set as the filtering threshold to ensure that only MFs which differ in the respective varieties with statistical significance are passed on and further processed (the lower the *p*-value, the more significant difference between the varieties). The final filtering step was conducted using fold change (FC) analysis. The value of FC was calculated as the MFs abundance ratios. MFs with FC value ≥ 16.0 and higher abundance compared with the other three species were picked out. Those selected MFs were re-extracted from the raw data files of the samples using recursive feature extraction to avoid any false positive results. Based on this approach, 292 MFs were selected as the marker compound candidates. A total of 12 markers were finally assigned and their identification information was summarized in Table 1.

3.4. Chemometric analysis

Principal component analysis (PCA), which was used to retain maximum variance of the data while reducing its dimensionality, was used as the data visualization tool in this study. Both positive and negative filtered data of 60 *M. cordata* samples from different

origins were subjected to PCA algorithm in the MPP software. As illustrated in Fig.3, a three-component PCA score plot of HPLC-Q-TOF/MS data was utilized depict general variation of *M. cordata* among four different origins. It was found that the classification of these four groups was clear. The three-component PCA model cumulatively accounted for 98% of variation. In other words, significant markers for *M. cordata* samples from different origins were found in positive ion mode but not in negative ion mode. The phenomenon indicated that the secondary metabolites in *M. cordata* from different origins had obviously changed.

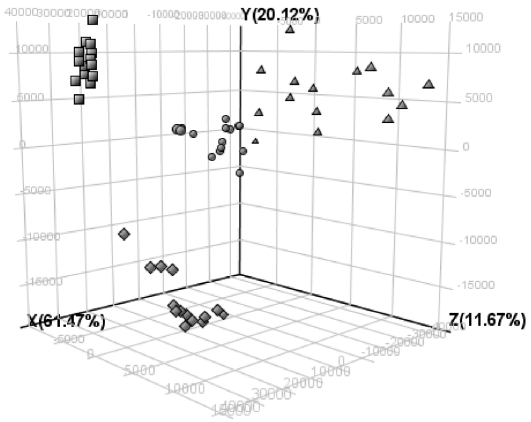

Fig.3 PCA scores plot and loading plot of JinZhai（▲), ChangSha（■), LouDi (◆) and QiMen (●) samples for positive ionization data.

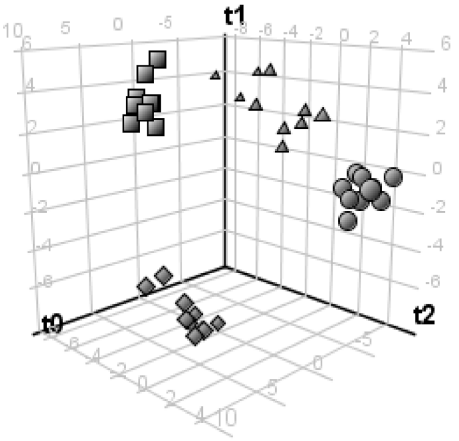

Fig.4 3D PLS-DA scores plot of JinZhai（▲), ChangSha（■), LouDi (◆) and Qi Men (●) samples for positive ionization data.

A widely used supervised pattern recognition method capable of sample class prediction, PLS-DA, was employed to further classify *M. cordata* from different origins. Ten samples from each origin were selected randomly for model training and the remaining was used for testing. Percentage of the right classification of both model training and validation was 100%, indicating that no sample was misclassified in the both

procedures. Good classification of *M. cordata* from four major different origins was obtained by PLS-DA and was illustrated in Fig.4.

3.5. Characterization of maker compounds

As discussed in section 3.3, 12 maker candidates were obtained. In order to determine the structural of potential biomarkers, the MS/MS fragmentation pathways were taken. Biomarker **3** eluted in 8.867 min and its *m/z* value was 206, was tentatively identified as *N*-methyl-7-demethoxyl-hydrocotarnine by the MS/MS fragmentation behaviors (Supplementary Fig. S1, see Supporting Information). The ion appeared at *m/z* 149, which corresponds to the retro-Diels-Alder (RDA) reaction of B-ring opening. The ion at *m/z* 190 and 119 were detected due to the loss of a methane molecular and CH_2O fragment from the ion at *m/z* 206 and 149, respectively. Biomarker **7** eluted in 16.626 min and exists only in Loudi, was identified as protopine-type alkaloid depending on RDA reaction and $[M-18]^+$ ion which were the characteristic MS/MS spectrum pattern for this type of alkaloid (Supplementary Fig. S1, see Supporting Information). The fragment ion at *m/z* 222 was observed corresponding to the RDA fragmentation reaction. The ion at *m/z* 206 was formed by the loss of a methane molecular from the ion at *m/z* 222, and the neutral elimination of a CH_2O fragment from the ion at *m/z* 206 lead to formation of the ion at *m/z* 176. The ions at *m/z* 366 and 135 were observed corresponding to the loss of a H_2O and CO molecular from the ion at *m/z* 384 and 163, respectively. Therefore, biomarker **7** was tentatively identified as 13-methylcryptopine. In addition to the marker compounds **3** and **7**, the others biomarker were also tentatively determined by the MS/MS fragmentation behaviors (Supplementary Fig. S1, see Supporting Information). The fragmentation pathways of biomarker **3** and **7** are proposed in Fig 5. which agree with

literature.[17-18]

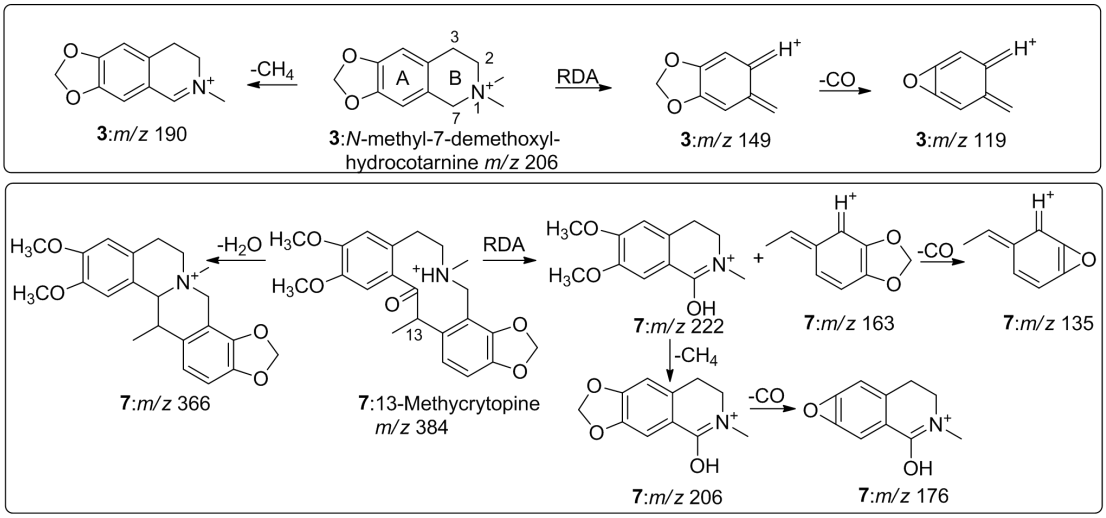

Fig.5 the proposed fragmentation pathways of biomarker compounds **3** and **7**.

4. Conclusions

HPLC-Q-TOF-MS combined with an "Xcharge C18" column was an efficient and convenient way for analyzing the polar and basic secondary metabolites. The result of PCA and PLS-DA has proved that it is possible to distinguish different *M. cordata* from various geographical origins. The marker compounds contributing to the discrimination were tentatively identified by the MS/MS fragmentation pathways. This method also can be applied for the analyzing of herb medicine that contained polar components and alkaloids.

Acknowledgements

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Table 1. the retention time (Rt), $[M+H]^+$, identification and distribution of marker compounds

No.	Rt (min)	$[M+H]^+$ (*m/z*)	Identification	Distribution
1	6.250	498.1384	Alkaloid	ChangSha
2	8.653	311.0927	Not alkaloid	QiMen > JinZhai, LouDi, ChangSha
3	8.657	206.0831	*N*-methyl-7-demethoxyl-hydrocotarnine	JinZhai> ChangSha> LouDi
4	11.890	610.1375	Quercetin derivative	ChangSha> JinZhai, LouDi, QiMen
5	13.331	360.1078	Alkaloid	ChangSha, LouDi > JinZhai, QiMen
6	13.406	452.0592	Sanguinarine derivative	ChangSha
7	16.604	384.1474	13-Methylcryptopine	LouDi
8	17.040	386.0513	6,10-dihydroxyl chelidonine	LouDi
9	19.477	371.2600	Not alkaloid	ChangSha
10	22.012	448.1764	Chelerythrine derivative	QiMen> JinZhai, ChangSha, LouDi
11	22.395	121.0303	Not alkaloid	ChangSha, LouDi > JinZhai, QiMen
12	22.395	279.1593	Not alkaloid	JinZhai> QiMen> ChangSha> LouDi

">" represent the level of biomarker of the former higher than the latter.

Captions:

Fig.1 TIC chromatogram on Zorbax Eclipse Plus C18 (a) and XCharge C18 (b).

Fig.2 UV chromatogram (280nm) on Zorbax Eclipse Plus C_{18} (a) and XCharge C_{18} (b).

Fig.3 PCA scores plot and loading plot of JinZhai（▲), ChangSha（■), LouDi (◆) and QiMen (●) samples for positive ionization data.

Fig.4 3D PLS-DA scores plot of JinZhai（▲), ChangSha（■), LouDi (◆) and QiMen (●) samples according to positive ionization mode data.

Fig.5 the proposed fragmentation pathways of biomarker compounds **2** and **7**.