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An experimental design approach using response surface techniques to obtain optimal liquid chromatography and mass spectrometry conditions to determine the alkaloids in *Meconopsi* species

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ABSTRACT

A statistic approach using response surface methodology (RSM) for optimization of the ultra-high performance liquid chromatography (UHPLC) gradient and ionization response of electrospray ionization mass spectrometry (ESI-MS) to analyze the main alkaloids from the plant matrices of six *Meconopsi* species is presented. The optimization was performed with Box–Behnken designs (BBD) and the multicriteria response variables were described using global Derringer's desirability. Four parameters of UHPLC and six major parameters of ESI-MS were investigated for their contribution to analytes separation and response, leading to a total of 27 and 54 experiments being performed for each instrument, respectively. Quantitative analysis of four main alkaloids in nine samples from six *Meconopsis* species was employed to evaluate the statistical significance of the parameters on UHPLC–QTOF/ESI-MS analytes response. The results indicated that the optimized UHPLC–QTOF-MS method is very sensitive with the limit of detections (LODs) ranging from 0.5 to 0.1 ng/ml. The overall intra-day and the inter-day variations were less than 2.45%. The recovery of the method was in the range of 94.3–104.8% with RSD less than 4.0%. This approach has important implication in sensitivity enhancement of the ultra-trace determination of alkaloids from complex matrixes in the fields of natural products, metabolomics and pharmacokinetics.

1. Introduction

The genus *Meconopsis* Vig., known as the "Blue poppy", belongs to the Papaveraceae family. There are a total of 49 species of the *Meconopsis* genus worldwide, and about 38 species distributed in the west of China, including Qinghai, Sichuan, Yunnan provinces and the Tibet region [1–2]. Most of *Meconopsis* species are strictly located in the high alpine lands and meadows of Qinghai-Tibet plateau at an altitude ranging from 3000 to 5000 m [1,3]. Many *Meconopsis* species, such as *M. integrifolia*, *M. torquata*, *M. horridula*, *M. racemosa* and *M. quintuplinervia*, have long been used as Tibetan folk remedy, and were recorded in Tibetan ancient medicinal literature (Yue Wang Yao Zhen, eighth century A.D.) and Flora of Tibet [1,3]. Among them, *M. horridula* and *M. racemosa* are used as traditional Tibetan medicine (named Cai-Wen) to clear away heat and reduce pain; *M. quintuplinervia*, *M. integrifolia*, *M. torquata* and *M. punicea* are used as traditional Tibetan medicine (named Wu-Ba-La) to clear away heat and toxic materials, and to act as anti-inflammatory and antipyretic agents [3]. Moreover, recent pharmacological studies revealed that the ethanol extract of *M. quintuplinervia* possesses remarkable analgesic effect and hepatic protective effect [4,5]. Previous phytochemical investigations have demonstrated that alkaloids [6–12] are the main bioactive components of *Meconopsis* plants. However, little information is available on the quality evaluation and standardization methods for both qualitative and quantitative determination of major components of *Meconopsis* plants. It is therefore necessary to develop a reliable analytical method to determine the safety and efficacy of traditional Tibetan herbs.

Currently, high performance liquid chromatography (HPLC) coupled with time-of-flight mass spectrometry (TOF-MS) has been used in composition analysis and quantification of a variety of natural product compounds [13–15]. Compared to traditional HPLC analysis, UHPLC analysis is of higher speed, improved sensitivity, selectivity and specificity. In addition, QTOF-MS allows the generation of mass information with greater accuracy and precision.

Although liquid chromatography–mass spectrometry (LC–MS) has proven to be a powerful tool in the comprehensive

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determination of multiple constituents in complex herbal extracts, it is still difficult to optimize the chromatographic conditions and the ionization response of electrospray ionization mass spectrometry (ESI-MS).

Since the alkaloids are physicochemically similar and not abundant in some *Meconopsis* species, their chromatographic separation may be problematic, and burdened by the differences and concentrations of other components. Furthermore, ammonia, which is commonly used as an additive in the mobile phase to improve peak shape and resolution for HPLC analysis of alkaloids, can inhibit the ionization efficiency of the alkaloids, thus resulting in decreased ESI-MS sensitivity. Since several factors are known to influence analyte response in ESI-MS, it is important to identify the key parameters that elicit major influence on analyte signal intensity and quality. For instance, the physicochemical properties of the liquid phase delivered from LC (surface tension, solution conductivity, *etc.*) are known to influence the formation of Taylor cone, the size of charged droplets and the evaporate–explosion rate of charged droplets [16–19]. High voltage applied at the tip of the capillary and sheath gas flow rate are also important factors affecting the efficiency of ionization. These parameters cannot be varied independently and should be optimized together.

Response surface methodology (RSM) is a widely used optimization approach for chromatographic study. Quadratic polynomial models have been considered as the most appropriate solution for building response surface to predict the optimized chromatographic conditions [20,21]. The most prevalent applications are centered on sample preparations, chromatographic conditions and absorbance detection sensitivity [22–28]. The prime advantage of RSM is the ability to acquire useful information about the system by conducting a minimal number of experiments without prior knowledge of the composition or physicochemical properties of the tested sample [21,29,30]. Box–Behnken designs (BBDs) are a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. They are more efficient than other response surface designs since they can be used to establish a quadratic response surface [20,21,30].

Generally, three steps are involved in RSM. The first step is a screening experiment to identify the important factors. Once the important independent variables are identified, it is necessary to determine if the current levels or settings of the independent variables result in a near optimal response. If not, a set of adjustments should be made to move the process towards the optimum. Finally, designed experiments for response surface methodology are performed to determine the optimum point. To do so, it is necessary for the polynomial function to contain quadratic terms according to the equation below:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where k is the number of variables, β_0 is the constant term, β_i, β_{ii} and β_{ij} represent the coefficient of the first order terms, quadratic terms and interaction terms, respectively, and ε is the residual associated to the experiments. To estimate the parameters in Eq. (1), the experimental design must insure that all studied variables are examined in at least three factor levels and that second-order symmetrical designs, such as three-level factorial design, BBDs, are adequate. In this study, BBDs were used for the optimization of chromatographic conditions and mass detection response due to their efficiency and flexibility.

In separation techniques, both separation and optimization goals, such as sensitivity, peak shape and analytical time, have to be considered. Therefore, the selection of optimal conditions requires a multicriteria decision making approach. Derringer's desirability function [31] has been used in many fields as a multicriteria decision making approach for the simultaneous optimization of several goals. The major advantage of Derringer's desirability is that if one of the criteria is not met, then the overall product will be unacceptable. Furthermore, this method offers the user flexibility in the definition of desirability functions. Therefore, the Derringer's desirability function was applied in selecting the optimum chromatographic and mass spectrometric conditions for quantitative determination of alkaloids in species of the *Meconopsi* genus. To date, some main MS parameters of an ion trap and a quadruple have been thoroughly optimized using RSMs [32–35]. Differing from other types of MS instruments, more factors affect the sensitivity and resolution of a QTOF instrument [36]. In this study, BBDs were selected for the evaluation of the chromatographic behavior and sensitivity of ESI-MS detection. The novelty comes from the improvement of the optimization steps accomplished by the application of Derringer's desirability function to optimize the gradient mobile phase of UHPLC and ESI response of QTOF-MS.

2. Experimental

2.1. Chemicals

Acetonitrile (ACN, HPLC–MS grade) was purchased from Fisher Scientific UK, (Loughborough, UK) and formic acid (spectroscopy grade) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Pure water was from a Milli-Q SP Regent Water system (Millipore, Bedford, MA, USA). Leucine-enkephalin was obtained from Sigma–Aldrich.

2.2. Sample preparation

Six *Meconopsi* species were collected in Qinghai province and the Tibet region of China in 2007. The plant material was identified by Mr. Suo-lang Gesang, Tibet Autonomous Region Institute for Food and Drug Control. The dry plant samples were grounded to fine powder by a pulverizer, and 0.2 g of powder was placed in a 10 ml capped conical flask and 2 ml methanol was added, and was extracted under supersonic washer (50 Hz) for 30 min. Then, the extract was filtered and the residue was extracted again for three times. The extraction solutions were combined, and diluted to 10 ml with methanol in a volumetric flask, and then filtered through a 0.22 μm PTFE syringe filter. An aliquot of each filtrate (2 μl) was injected into the UHPLC instrument for analysis. Eight reference compounds, namely tricin (**1**), hydnocarpin (**2**), *O*-methylflavinantine (**3**), oleracein E (**4**), mecambridine (**5**), protopine (**6**), alborine (**7**) and berberine (**8**) (Fig. 1) were isolated from *Meconopsi integrifolia* and were identified based on IR, UV, NMR spectroscopy analysis alongwith literature data. They were dissolved in methanol to give a concentration of 0.1–5 μg/ml.

2.3. Liquid chromatography

UHPLC was performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent delivery system, autosampler, and a photodiode-array detection (DAD) system. The chromatography was performed on a Waters Acquity BEH C_{18} column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA, USA). The mobile phase consisted of (A) 0.2% ammonia in water and (B) ACN. The UHPLC eluting conditions were optimized as follows: linear gradient from 2% to 45% B (0–5 min), linear gradient from 45% to 81% B (5–11.1 min), linear gradient from 81% to 95% B (11.1–13 min), and linear gradient from 95% to 2% B (13–14 min). The flow rate was 0.5 ml/min. The column and autosampler were maintained at 35 and 10 °C, respectively. Each wash cycle consisted of 200 μl of

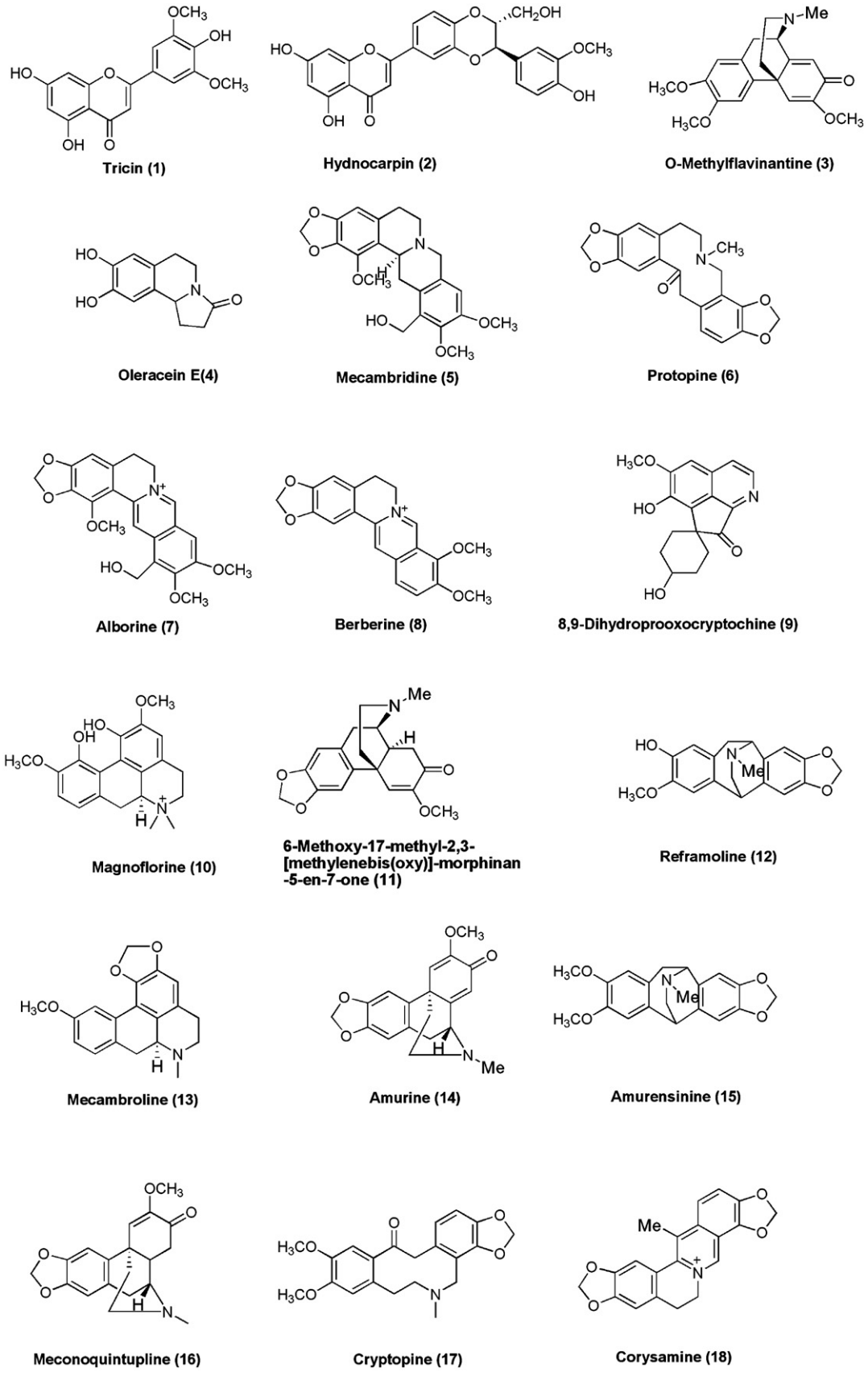

Fig. 1. Structures assigned in the extracts of *Meconopsis* species.

Table 1
Multicriteria decision making criteria and desirability limits for the simultaneous optimization of variables on UHPLC elution gradient and mass detection response through overall desirability response.

Variables optimization and Derringer desirability function	Individual response	Optimization criteria/desirability limits
ACN Content and time of elution gradient $D_{uplc} = (d_1 \times d_2 \times d_3 \times d_4 \times d_5)^{1/5}$	d_i = resolution between the specified peak and its adjacent peaks (RA_i, i = 1–3)	$d_i = \begin{cases} (RA_i - 0.5 \times 1.5)/1.5\,(1.5 - 0.6) \\ 0 \text{ if } RA_i < 0.5 \times 1.5 \\ 1 \text{ if } RA_i > 1.5 \end{cases}$
	d_4 = retention time (Rt) of the last peak	$d_4 = \begin{cases} Rt/15\,(Rt \le 15) \text{ or } 15/Rt\,(Rt > 15)(1 - 0.5) \\ 0 \text{ if } Rt < 5 \text{ or } Rt > 20 \\ 1 \text{ if } Rt = 15 \end{cases}$
	d_5 = number of total determined peaks (Tps)	$d_5 = \begin{cases} (Tps - 0.5Tps_{(max)})/0.5Tps_{(max)}(1 - 0.5) \\ 0 \text{ if } Tps < 0.5\,Tps_{(max)} \\ 1 \text{ if } Tps = Tps_{(max)} \end{cases}$
Parameters on QTOF-MS peak response $D_{MS} = (RS1 \times RS2 \times RSs)^{1/3}$	RSi = peak area of three specified compounds (PAi, I = 1–3)	$RSi = \begin{cases} (PAi - 0.5PA_{(max)})/0.5PA_{(max)}(1 - 0.5) \\ 0 \text{ if } PAi \le 0.5PA_{(max)} \\ 1 \text{ if } PAi = PA_{(max)} \end{cases}$

strong solvent (80% ACN) and 600 μl of weak solvent (10% ACN). The injection volume of the standards and sample was 2 μl.

2.4. Mass spectrometry

Mass spectrometry was performed using a Waters QTOF Premier (Micromass MS Technologies, Manchester, UK) operating in positive ion mode. The nebulization gas was set to 550 l/h at 400 °C, and the source temperature set to 101 °C. The capillary voltage and cone voltage were set to 3000 and 40 V, respectively. The Argon was employed as the collision gas at a pressure of 7.066×10^{-3} Pa. The instrument was operated with the first resolving quadrupole in a wide pass mode with the collision cell operating at 5 V. The molecular masses were accurately determined with reference compound Leucine-enkephalin in the LockSpray mode (m/z 556.2771) at a concentration of 100 pg/μl and an infusion flow rate of 10 μl/min. The data was collected into two separate data channels, with the instrument spending 0.2 s on data acquisition for each channel with a 0.02 s inter-channel delay.

2.5. Experimental design

Data analysis and desirability function calculations were performed by using Microsoft Excel 2002 (Microsoft Corporation). Experimental design was performed by using SAS® 9.0 (SAS Institute Inc., Cary, USA). Optimization criteria and desirability limits were summarized in Table 1. Four factors of UHPLC were optimized by BBD first, including, (1) gradient time T_I (min); (2) ratio of mobile phase B ACN_I (B%) for the first solvent gradient; (3) gradient time T_II (min) and (4) ratio of mobile phase B ACN_II (B%) for the second solvent gradient. The mobile phase of initial gradient was 2% of B, and a gradient was performed according to each designed experiment in Table 2: linear gradient from 2% to ACN_I (B%) (0 to T_I, min), linear gradient from ACN_I (B%) to ACN_II

Table 2
Plan of experiment for Box–Behnken design and individual and global Derringer desirability of UHPLC optimization.

Experiment	Factors[a]				Desirability[b]					
	ACN_I (%)	T_I (min)	ACN_II (%)	T_II (min)	d_1	d_2	d_3	d_4	d_5	D_{uplc}
1	20	3	80	11.5	0.947	0.699	0.908	0.947	0.933	0.900
2	20	7	80	11.5	0.532	0.000	0.000	0.529	0.303	0.000
3	60	3	80	11.5	0.912	0.000	0.742	0.995	0.506	0.000
4	60	7	80	11.5	1.000	0.637	0.000	0.945	0.596	0.000
5	40	5	60	8.0	0.958	0.735	0.965	0.978	0.888	0.916
6	40	5	60	15.0	1.000	0.000	0.000	0.963	0.663	0.000
7	40	5	100	8.0	0.922	0.000	1.000	0.938	0.483	0.000
8	40	5	100	15.0	0.985	0.668	0.940	0.939	0.888	0.895
9	20	5	80	8.0	0.942	0.000	1.000	0.875	0.416	0.000
10	20	5	80	15.0	1.023	0.806	0.000	0.921	0.303	0.000
11	60	5	80	8.0	1.000	0.962	1.000	0.968	1.000	0.988
12	60	5	80	15.0	0.622	0.000	0.989	0.910	0.753	0.000
13	40	3	60	11.5	0.347	0.512	0.000	0.646	0.371	0.000
14	40	3	100	11.5	1.000	0.000	1.000	0.917	0.663	0.000
15	40	7	60	11.5	0.837	0.000	0.000	0.580	0.281	0.000
16	40	7	100	11.5	0.977	0.000	0.650	0.581	0.169	0.000
17	20	5	60	11.5	0.830	0.990	0.000	0.547	0.416	0.000
18	20	5	100	11.5	0.770	1.000	0.000	0.799	0.663	0.000
19	60	5	60	11.5	1.000	0.000	0.000	0.662	0.258	0.000
20	60	5	100	11.5	1.000	0.925	0.964	0.997	1.000	0.981
21	40	3	80	8.0	1.000	0.880	0.000	0.962	0.820	0.000
22	40	3	80	15.0	0.989	0.000	0.640	0.986	0.708	0.000
23	40	7	80	8.0	0.970	0.000	0.000	0.610	0.258	0.000
24	40	7	80	15.0	1.000	0.000	0.000	0.000	0.990	0.596
25	40	5	80	11.5	1.000	0.924	1.000	1.000	0.925	0.865
26	40	5	80	11.5	1.000	0.890	0.985	0.972	0.926	0.865
27	40	5	80	11.5	1.000	0.919	1.000	0.979	0.925	0.865

[a] Factors of ACN_I and T_I, represent concentration of acetonitrile at first time point; ACN_II and T_II represent concentration of acetonitrile at second time point.
[b] d_1–d_5 and D_{uplc} represent the individual responses and global Derringer desirability of UHPLC optimization, which are the same as in Table 1.

Table 3
Factors, levels, individual and global Derringer desirability of mass detection responses by Box–Behnken design.

Run no.	Factors[a]						Responses[b]			
	CAV	COV	ST	DT	DF	NH_3	*RS*1	*RS*2	*RS*3	D_{MS}
01	2.5	30	105	300	600	0.25	0.313	0.000	0.444	0.000
02	2.5	30	105	400	600	0.25	0.738	0.548	0.734	0.667
03	2.5	50	105	300	600	0.25	0.000	0.000	0.463	0.000
04	2.5	50	105	400	600	0.25	0.538	0.579	0.746	0.615
05	3.5	30	105	300	600	0.25	0.000	0.000	0.000	0.000
06	3.5	30	105	400	600	0.25	0.428	0.154	0.466	0.313
07	3.5	50	105	300	600	0.25	0.000	0.000	0.000	0.000
08	3.5	50	105	400	600	0.25	0.331	0.127	0.544	0.283
09	3.0	30	90	350	550	0.25	0.529	0.000	0.764	0.000
10	3.0	30	90	350	650	0.25	0.639	0.000	0.819	0.000
11	3.0	30	120	350	550	0.25	0.639	0.000	0.819	0.000
12	3.0	30	120	350	650	0.25	0.653	0.000	0.761	0.000
13	3.0	50	90	350	550	0.25	0.507	0.000	0.891	0.000
14	3.0	50	90	350	650	0.25	0.529	0.387	0.902	0.570
15	3.0	50	120	350	550	0.25	0.507	0.000	0.815	0.000
16	3.0	50	120	350	650	0.25	0.456	0.000	0.768	0.000
17	3.0	40	90	300	600	0.10	1.000	0.667	0.894	0.842
18	3.0	40	90	300	600	0.40	0.000	0.000	0.000	0.000
19	3.0	40	90	400	600	0.10	0.714	1.000	0.836	0.842
20	3.0	40	90	400	600	0.40	0.000	0.482	0.429	0.000
21	3.0	40	120	300	600	0.10	0.000	0.000	0.000	0.000
22	3.0	40	120	300	600	0.40	0.000	0.000	0.000	0.000
23	3.0	40	120	400	600	0.10	0.000	0.309	0.437	0.000
24	3.0	40	120	400	600	0.40	0.000	0.432	0.408	0.000
25	2.5	40	105	300	550	0.25	0.000	0.000	0.508	0.000
26	3.5	40	105	300	550	0.25	0.000	0.000	0.000	0.000
27	2.5	40	105	300	650	0.25	0.000	0.000	0.505	0.000
28	3.5	40	105	300	650	0.25	0.000	0.000	0.000	0.000
29	2.5	40	105	400	550	0.25	0.574	0.983	0.897	0.797
30	3.5	40	105	400	550	0.25	0.964	0.647	1.000	0.854
31	2.5	40	105	400	650	0.25	0.726	0.793	0.928	0.811
32	3.5	40	105	400	650	0.25	0.532	0.518	0.6500	0.564
33	3.0	30	105	350	550	0.10	0.000	0.000	0.000	0.000
34	3.0	50	105	350	550	0.10	0.000	0.000	0.000	0.000
35	3.0	30	105	350	550	0.40	0.000	0.000	0.000	0.000
36	3.0	50	105	350	550	0.40	0.000	0.000	0.000	0.000
37	3.0	30	105	350	650	0.10	0.000	0.000	0.000	0.000
38	3.0	50	105	350	650	0.10	0.000	0.000	0.000	0.000
39	3.0	30	105	350	650	0.40	0.000	0.000	0.000	0.000
40	3.0	50	105	350	650	0.40	0.000	0.000	0.000	0.000
41	2.5	40	90	350	600	0.10	0.000	0.314	0.381	0.000
42	2.5	40	120	350	600	0.10	0.000	0.130	0.362	0.000
43	3.5	40	90	350	600	0.10	0.506	0.681	0.411	0.521
44	3.5	40	120	350	600	0.10	0.000	0.000	0.000	0.000
45	2.5	40	90	350	600	0.40	0.000	0.278	0.000	0.000
46	2.5	40	120	350	600	0.40	0.000	0.281	0.343	0.000
47	3.5	40	90	350	600	0.40	0.000	0.100	0.000	0.000
48	3.5	40	120	350	600	0.40	0.000	0.103	0.000	0.000
49	3.0	40	105	350	600	0.25	0.000	0.204	0.493	0.000
50	3.0	40	105	350	600	0.25	0.361	0.247	0.551	0.366
51	3.0	40	105	350	600	0.25	0.313	0.265	0.523	0.351
52	3.0	40	105	350	600	0.25	0.343	0.241	0.586	0.365
53	3.0	40	105	350	600	0.25	0.288	0.173	0.508	0.294
54	3.0	40	105	350	600	0.25	0.337	0.226	0.541	0.345

[a] Abbreviations of factors are the same as in text.
[b] Abbreviations of *RS*1, *RS*2, *RS*3 and D_{MS} represent the individual response and global Derringer desirability of mass sensitivity, respectively, which are the same as in Table 1.

(B%) (T_I to T_II, min), linear gradient from ACN_II (B%) to 100% B (T_II-13 min), and linear gradient from 100% to 2% B (13–14 min). Then, seven factors of ESI response of QTOF-MS, including capillary voltage (CAV), sample cone voltage (COV), desolvation temperature (DT), source temperature (ST), desolvation gas flow rate (DF) and concentration of ammonia (NH_3) were optimized by BBD. When performing this optimization step, the mobile phase composition and elution gradient was the same as that described in Section 2.3; only the content of ammonia varied according to each designed experiment (a total 54 experiments were assayed, see Table 3 for experimental design details).

3. Results and discussion

3.1. Optimization of chromatographic conditions

The independent variables were defined during the preliminary study, but some common parameters such as column temperature and flow rate were excluded, since they can be easily predicted by experience and chromatographic theory knowledge. The concentration of ammonia is one of the factors affecting the separation of alkaloids. Preliminary results showed that the peaks were tailing and the separation efficiency was unsatisfactory when the

content of ammonia was lower than 0.1%. The peak shape improved when the content of ammonia was higher than 0.2%. Moreover, the preliminary experiments indicated that the mobile phase of acetonitrile gave better peak shape, lower system pressure and shorter running time than that of methanol. Therefore, acetonitrile and 0.2% ammonia–H_2O were selected to be the mobile phase in subsequent studies.

Based on the preliminary experiments, two steps of gradient profiles were investigated. A total of 27 experiments were assayed by simultaneously varying the gradient step with the initial mobile phase of aqueous solution containing 0.2% ammonia. Responses considered to be relevant in the optimization were combined in the Derringer desirability function shown in Table 2. The initial composition of mobile phase was 2% of B, and the end point of the first linear gradient was optimized by varying B from 20% to 60% within 3–7 min. This range allowed a complete separation of the flavonoids, such as tricin (**1**), hydnocarpin (**2**). The resolution of four major alkaloids was optimized on the second end point of the gradient step from 60% to 100% of B within 8–15 min. The effect of factors on the global desirability response was shown in Fig. 2. There are three criteria selected for optimization: (1) resolutions between the target compounds **3**, **6** and **5** and their corresponding adjacent peaks (d_1–d_3 in Table 1); (2) retention time of the last peak in each chromatogram (d_4); and (3) the number of total determined peaks (d_5). The best optimal gradient condition, which resulted in the best resolution within a short analysis time, could be obtained as the global desirability response reaches its maximum. For the initial gradient step, in which B varied from 2% to 45% in 5 min, and the second gradient step, in which B varied from 45% to 81% in 5–11.1 min as shown in Fig. 2. After this second step, the ACN percentage was linearly increased up to 95% in 2 min for a complete column wash. The optimized condition was thus used for the analysis of the nine samples from six *Meconopsi* species.

3.2. Optimization of response of mass detection

Since contents of alkaloids in the studied plants are not abundant and their intensity responses are different, it is necessary to evaluate the influence of instrument parameters on analytes response during ESI-MS experiments to increase the ESI sensitiv-

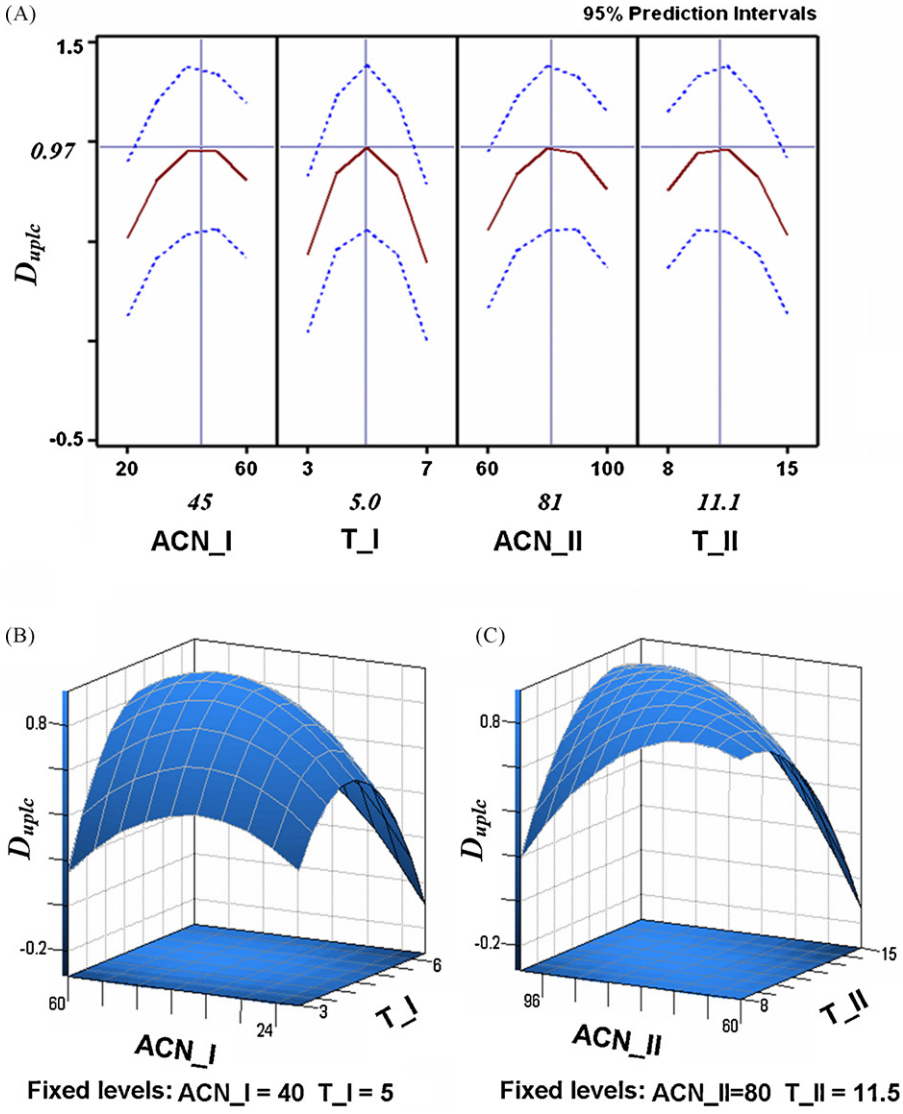

Fig. 2. The main effect plots of independent factors on global multicriteria desirability (panel A), and response surface plots representing the global multicriteria desirability as a function of: (panel B) content of acetonitrile and gradient time for optimizing the first point by keeping the content of ACN (ACN_I) at first point of 40% and the gradient time (T_I) at first point of 5.0 min; (panel C) content of acetonitrile and gradient time for optimizing the second point by keeping the content of ACN (ACN_II) at second point of 80% and the gradient time (T_II) at second point of 11.5 min. Abbreviations: ACN_I, ACN_II, T_I and T_II were the same as in Table 2; the same as in Table 1 global multicriteria desirability of responses (D_{uplc}).

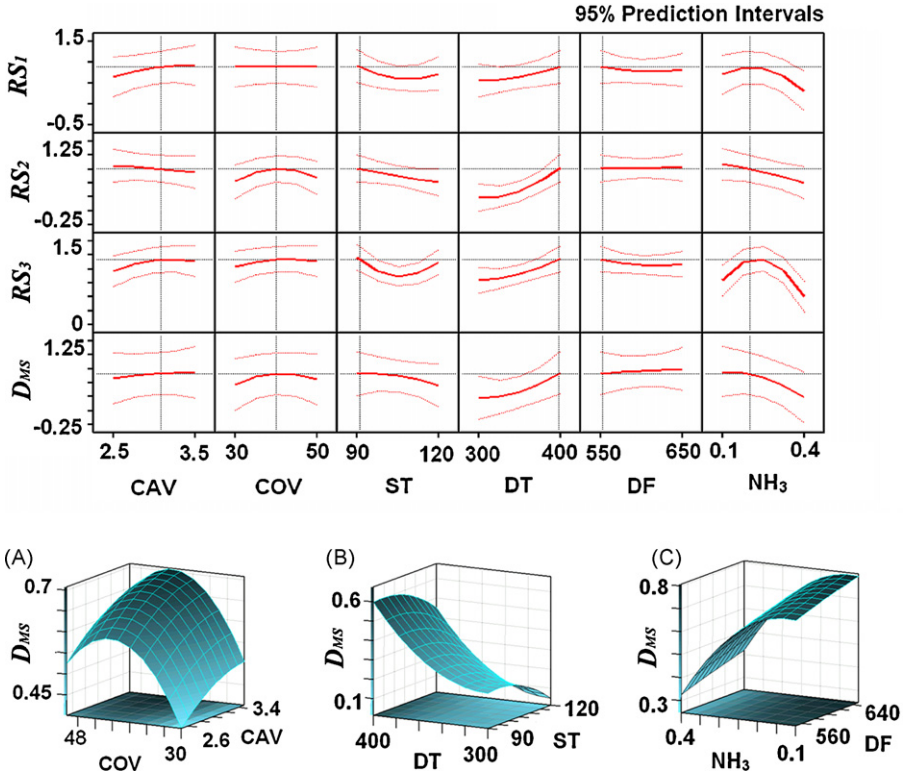

Fig. 3. The main effect plots of independent factors on response of compound **3** (*RS*1), compound **6** (*RS*2), response of compound **5** (*RS*3) and global multicriteria desirability (D_{MS}, upper panel) and response surface plots representing the global multicriteria desirability (D_{MS}, lower panel) as a function of capillary voltage (CAV) vs sample cone voltage (COV, A), desolvation temperature (DT) vs source temperature (ST, B) and concentration of ammonia (NH_3) vs desolvation gas flow rate (DF, C) for the optimization of maximum response of peak intensity.

ity of trace level alkaloid detection. As stated previously, many factors influence the ESI sensitivity, not only the parameters of mass spectrometer, but also the composition of LC mobile phase [35–37]. These parameters were chosen based on the significance of their influence on the ESI process, and were further screened from the initial experimental trials. For example, flow rate is one of the important factors that were critical for UHPLC separation. However, no significant effect was observed in our initial experiments when flow rate varied from 0.4 to 0.6 ml/min. In addition, the ESI on a QTOF-MS can be performed at a higher flow rate (typically 300–500 μl/min) by directing a gas flow into the effluent stream [35]. Therefore based on the above guidelines, six parameters, namely capillary voltage (CAV), sampling cone voltage (COV), source temperature (ST), desolvation temperature (DT), desolvation gas flow rate (DF) and the concentration of ammonia (NH_3) were selected for optimization. The levels of each instrumental

Table 4
Regression equation, correlation coefficients, linearity ranges, limit of detection (LODs), quantitation (LOQs), precision and recovery for the four markers of *Meconopsis* samples.

No.	Compounds	Regression equation	Linear range (μg/ml)	r^2	LODs (ng/ml)	LOQs (ng/ml)	Intra-day (RSD, %, $n=6$)	Inter-day (RSD, %, $n=6$)	Recovery (%, $n=5$)
1	*O*-Methylflavinantine (**3**)	$y=15.64x+60.45$	0.40–4.0	0.9984	0.5	5	0.98	2.45	94.3
3	Mecambridine (**5**)	$y=415.0x+3.923$	0.07–0.7	0.9975	0.3	3	1.05	1.66	96.6
4	Protopine (**6**)	$y=14.28x+47.34$	0.52–41.6	0.9982	0.1	0.5	0.50	2.06	104.8
5	Albore (**7**)	$y=12.5x+90.71$	0.01–1.0	0.9994	0.2	1	0.52	2.33	95.1

Table 5
The contents of four standard compounds in the collected *Meconopsis* samples (μg/g).

Samples no.	Species	Location of collection	Contents (μg/g) ± RSD% ($n=5$)			
			O-Methylflavinantine (**3**)	Mecambridine (**5**)	Protopine (**6**)	Albore (**7**)
LRH-1	*Meconopsis torquata*	Ya-Dong Tibet	nd	nd	100.72 ± 3.38	tr
LRH-2	*Meconopsis racemosa*	Chang-Du Tibet	tr	tr	103.42 ± 3.20	tr
LRH-3	*Meconopsis integrifolia*	Dui-Long Tibet	nd	nd	106.93 ± 1.10	tr
LRH-4	*Meconopsis quintuplinervia*	Yushu Qinghai	46.34 ± 2.57	1.66 ± 3.93	58.78 ± 4.00	tr
LRH-5	*Meconopsis betonicifolia*	Lin-Zhi Tibet	nd	nd	37.81 ± 4.99	0.31 ± 2.86
LRH-6	*Meconopsis quintuplinervia*	Lulang Tibet	54.48 ± 3.74	1.11 ± 4.89	35.78 ± 3.36	tr
LRH-7	*Meconopsis horridula*	Lulang Tibet	tr	nd	178.71 ± 2.22	
LRH-8	*Meconopsis integrifolia*	Guo-Luo Qinghai	tr	nd	101.73 ± 3.81	tr
LRH-9	*Meconopsis horridula*	Yushu Qinghai	49.25 ± 1.85	1.94 ± 1.73	92.56 ± 3.20	tr

nd: Not detected; tr: Trace.

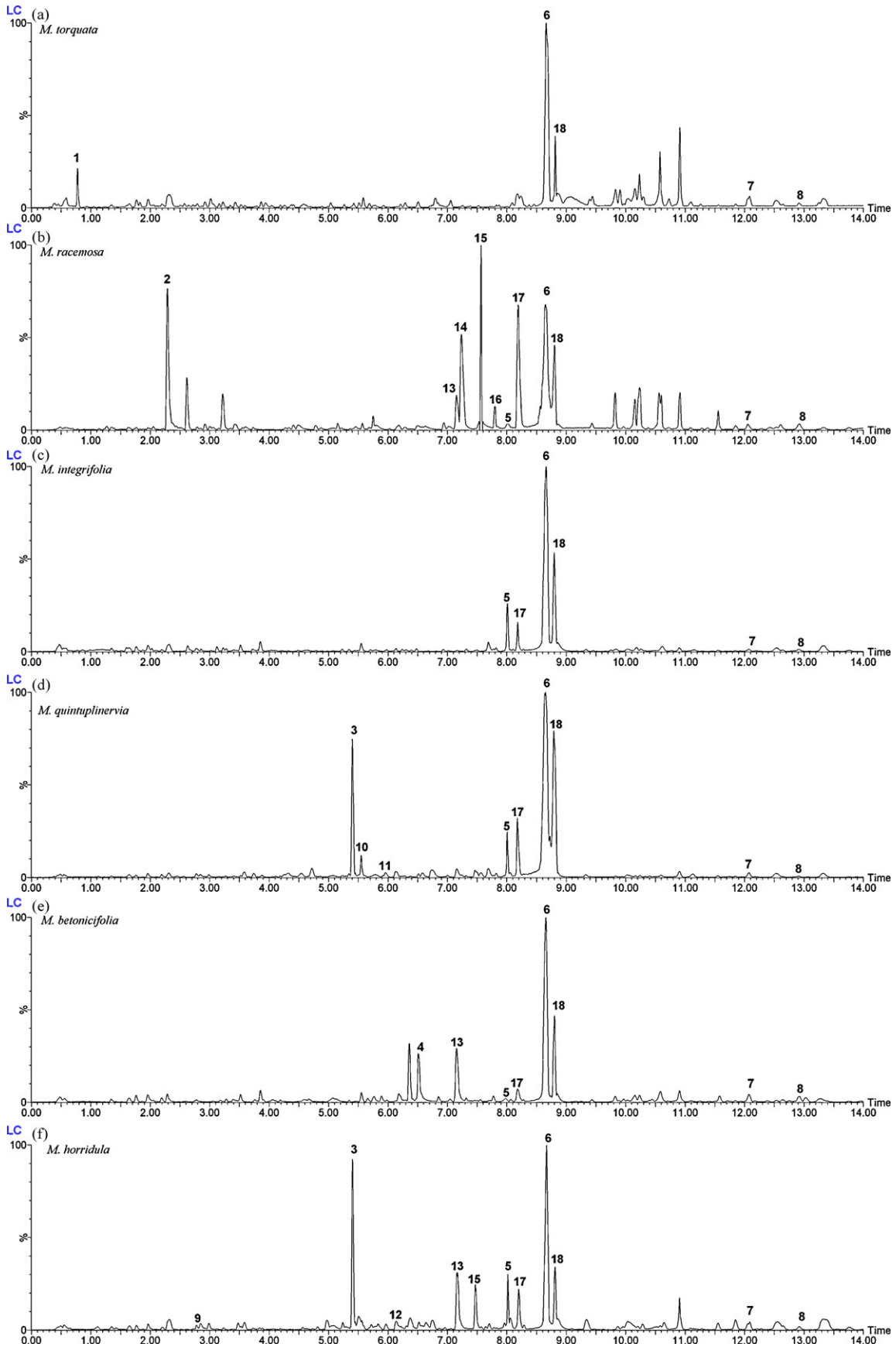

Fig. 4. UHPLC–QTOF BPI chromatograms of six *Meconopsis* species for identification: (a–f) BPI of ESI(+) TOF-MS from *M. torquata*; *M. racemosa*; *M. integrifolia*; *M. quintuplinervia*; *M. betonicifolia*; *M. horridula*.

Table 6
Element constituents of major ions observed in online positive UHPLC–MS spectra of the components identified from the *Meconopsis* species.

Peaks	Retention time	Compounds	Formula	Calculated	Observed	Error (ppm)
1	1.27	Tricin (**1**)	$C_{17}H_{15}O_7$	331.0818	331.0815	−0.9
			$C_{16}H_{11}O_7$	315.0505	315.0509	1.3
			$C_{15}H_{11}O_6$	287.0556	287.0561	1.7
			$C_{15}H_{10}O_5$	270.0528	270.0532	1.5
			$C_{14}H_{10}O_5$	258.0528	258.0523	−1.9
2	2.28	Hydnocarpin (**2**)	$C_{25}H_{21}O_9$	465.1186	465.1193	1.5
			$C_{25}H_{19}O_8$	447.1080	447.1078	−0.4
			$C_{24}H_{19}O_8$	435.1080	435.1067	−3.0
			$C_{15}H_{10}O_6$	286.0477	286.0476	−0.3
			$C_{14}H_9O_5$	257.0450	257.0446	−1.6
3	5.39	*O*-Methylflavinantine (**3**)	$C_{20}H_{24}NO_4$	342.1705	342.1703	−0.6
			$C_{18}H_{16}O_4$	296.1049	296.1050	0.3
			$C_{17}H_{17}O_4$	285.1127	285.1132	1.8
			$C_{18}H_{16}O_3$	280.1099	280.1095	−1.4
			$C_{16}H_{17}O_3$	257.1178	257.1183	1.9
4	6.51	Oleracein E (**4**)	$C_{12}H_{14}NO_3$	220.0974	220.0973	−0.5
			$C_{12}H_{12}NO_3$	218.0817	218.0819	0.9
			$C_8H_9O_2$	137.0603	137.0607	2.9
5	8.06	Mecambridine (**5**)	$C_{22}H_{26}NO_6$	400.1760	400.1754	−1.5
			$C_{12}H_{16}NO_2$	206.1181	206.1184	1.4
			$C_{11}H_{13}NO_2$	191.0946	191.0950	2.1
			$C_9H_{12}N$	134.0970	134.0973	2.2
6	8.80	Protopine (**6**)	$C_{20}H_{20}NO_5$	354.1341	354.1346	1.4
			$C_{12}H_{16}NO_2$	206.1181	206.1186	2.4
			$C_{11}H_{13}NO_2$	191.0946	191.0951	2.7
7	12.1	Alborine (**7**)	$C_{22}H_{22}NO_6$	396.1447	396.1448	0.3
			$C_{21}H_{18}NO_6$	380.1134	380.1130	−1.1
			$C_{21}H_{20}NO_3$	366.1341	366.1346	1.4
			$C_{20}H_{17}NO_4$	335.1158	335.1154	−1.2
8	13.1	Berberine (**8**)	$C_{20}H_{18}NO_4$	336.1236	336.1234	−0.6
			$C_{19}H_{14}NO_4$	320.0923	320.0920	−0.9
			$C_{18}H_{12}NO_4$	306.0766	306.0772	2.0
			$C_{18}H_{14}NO_3$	292.0974	292.0978	1.4
			$C_{17}H_{12}NO_3$	278.0817	278.0820	1.1
9	2.78	8,9-Dihydroprooxocryptochine (**9**)	$C_{17}H_{17}NO_5$	300.1236	300.1239	1.0
			$C_{17}H_{16}NO_3$	282.1130	282.1124	−2.1
			$C_{16}H_{12}NO_2$	250.0868	250.0863	−2.0
10	5.40	Magnofloring (**10**)	$C_{20}H_{24}NO_4$	342.1705	342.1706	0.3
			$C_{19}H_{22}NO_2$	296.1651	296.1658	2.4
			$C_{19}H_{22}NO$	280.1701	280.1707	2.1
			$C_{18}H_{19}NO$	265.1467	265.1462	−1.9
			$C_{15}H_{16}NO$	226.1232	226.1238	2.6
11	5.96	6-Methoxy-17-methyl-2,3-[methylenebis(oxy)]-morphinan-5-en-7-one (**11**)	$C_{19}H_{22}NO_4$	328.1549	328.1541	−2.4
			$C_{18}H_{18}NO_4$	312.1236	312.1243	2.2
			$C_{16}H_{15}O_4$	271.0970	271.0975	1.8
			$C_{15}H_{13}O_2$	225.0916	225.0908	−3.6
12	6.14	Reframoline (**12**)	$C_{19}H_{19}NO_5$	326.1392	326.1383	−2.8
			$C_{19}H_{17}O_4$	309.1127	309.1120	−2.3
			$C_{18}H_{16}NO_2$	278.1181	278.1173	−2.9
13	7.17	Mecambroline (**13**)	$C_{18}H_{17}NO_3$	296.1287	296.1285	−0.7
			$C_{17}H_{16}NO_2$	266.1181	266.1185	1.5
			$C_{17}H_{17}NO$	251.1310	251.1304	2.4
			$C_{14}H_{11}O$	195.0810	195.0809	−0.5
14	7.24	Amurensine (**14**)	$C_{19}H_{20}NO_4$	326.1392	326.1388	−1.2
			$C_{10}H_{12}NO_2$	178.0868	178.0872	2.2
15	7.56	Amurensinine (**15**)	$C_{20}H_{22}NO_4$	340.1549	340.1547	−0.6
			$C_{20}H_{19}O_4$	323.1283	323.1291	−3.1
			$C_{18}H_{16}NO_2$	278.1181	278.1176	−1.8
			$C_{17}H_{14}NO$	248.1075	248.1068	−2.8
16	7.81	Meconoquintupline (**16**)	$C_{19}H_{22}NO_4$	328.1549	328.1546	−0.9
			$C_{17}H_{18}NO_2$	268.1338	268.1343	1.9
17	8.20	Cryptopine (**17**)	$C_{21}H_{23}NO_5$	370.1654	370.1659	1.4
			$C_{20}H_{22}NO_3$	324.1600	324.1604	1.2
			$C_{12}H_{14}NO_2$	204.1025	204.1020	−2.4
			$C_{11}H_{12}NO_2$	190.0868	190.0861	−2.6

Table 6 (*Continued*)

Peaks	Retention time	Compounds	Formula	Calculated	Observed	Error (ppm)
18	8.65	Corysamine (**18**)	$C_{20}H_{16}NO_4$	334.1079	334.1073	−1.8
			$C_{19}H_{16}NO_3$	306.1130	306.1128	−0.7
			$C_{19}H_{14}NO_2$	288.1025	288.1031	2.1
			$C_{18}H_{14}NO_2$	276.1025	276.1029	1.4
			$C_{18}H_{14}NO$	260.1075	260.1069	−2.3
			$C_{17}H_{14}NO$	248.1075	248.1080	2.0
			$C_{17}H_{12}N$	230.0970	230.0976	2.6

parameter, namely CAV, COV, ST, DT and DF were determined by preliminary experimental results and instrumental limitation. The ammonia concentration varied from 0.1% to 0.4%, which covered the optimized concentration in the mobile phase. Three main alkaloids, namely *O*-methylflavinantine (**3**), mecambridine (**5**) and protopine (**6**) were selected as model compounds for optimization. A total of 54 experiments were assayed (see Table 3 for experimental design details).

Response surface modeling visualizes the dependence of a fitted response on two or more factors. The prediction profile allows the determination of a set of parameters, among different combinations that may cause similar effects, to optimize multiple responses. Fig. 3 illustrates the variations of the individual responses (*RS*1, *RS*2 and *RS*3) of three selected alkaloids and the global multicriteria desirability (D_{MS}) for the three compounds as functions of the six selected parameters. The effects of the selected parameters on Derringer's desirability (D_{MS}) as evaluated by response surface analysis were also demonstrated. The global multicriteria desirability (D_{MS}) was decreased as the concentration of ammonia increased and the optimized concentration of ammonia was at 0.1–0.2 %. However, as shown in Fig. 3, the response of compound **6** was different from those of compounds **3** and **5**. The response of compound **6** (*RS*2) varied linearly with respect to factors CAV, ST, DT and NH_3, but the responses of compounds **3** (*RS*1) and **5** (*RS*3) varied quadratically with respect to factors CAV, ST, DT and NH_3. Since the contents of compounds **3** and **5** in the plants were much lower than that of compound **6**, the optimal concentration of ammonia was at 0.2%. This concentration was also an optimized value for UHPLC separation, therefore, the final condition was set as CAV: 3.0 kV, COV: 40 V, ST: 90 °C, DT: 400 °C, DF: 550 l/min and ammonia concentration: 0.2%.

3.3. Sensitivity and validation of UHPLC–QTOF-MS method for quantitative analysis the alkaloids

Quantification of four major alkaloids, *O*-methylflavinantine (**3**), mecambridine (**5**), protopine (**6**) and alborine (**7**) in nine samples of six *Meconopsis* species has been used to evaluate the sensitivity and precision of the optimized UHPLC–QTOF-MS method. The quantification was carried out under full-scan conditions using extracted ion chromatograms (XICs) with a 50 mDa window of the protonated molecules. All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (y) versus concentrations (x, μg/ml) of the three markers in the standard solution at six different concentrations. The linearity of these standard curves is adequate, and the R^2 values of the three markers are from 0.9975 to 0.9994.

The sensitivity of the method was evaluated by determining the limits of detection (LODs) and limits of quantitation (LOQs). The LODs and LOQs were defined as each compound's signal to baseline noise peak ratio at a high of 3 and 10, respectively. These parameters were determined empirically by triplicate analysis of a series of decreasing concentrations of standard solution. The developed method was very sensitive with LODs below 0.5 ng/ml. Notably, the LODs were far lower than those in our previous work. For example, the LOD of protopine (**6**) is over one order of magnitude lower than the LOD of 1 ng/ml obtained with LC–MS instrument [37].

Method precision was checked by intra-day and inter-day variability. The intra-day variation was evaluated by determining a standard solution six consecutive times a day. The inter-day variation was conducted for three successive days using the same solution. The relative standard deviation (RSD) was taken as a measure of precision. The developed method was found to be precise, with the intra-day variability RSD values between 0.50% and 1.05% and the inter-day variability RSD values between 1.66% and 2.45%, as shown in Table 4.

Recovery was carried out by spiking accurate amounts of the four standards into sample 2, and then extracting and analyzing them under the proposed method. Each sample was analyzed in six replicates. The total amount of each analysis was calculated from the corresponding calibration curve. The recovery of the method was in the range of 94.3–104.8%, with RSD less than 4%.

The adequate sensitivity and precision of the results indicated method feasibility for determining crude plant samples, thus the optimized LC-QTOF–MS method was subsequently applied to the simultaneous quantitative analysis of the four alkaloids in nine samples of six *Meconopsis* species. The contents of the three marker compounds in each sample were analyzed by a regression equation, as shown in Table 5. The contents of protopine (**6**) were high in most *Meconopsis* samples, and the contents of *O*-methylflavinantine (**3**) were high in *M. quintuplinervia* (LRH4 and LRH 6) and *M. horridula* (LRH-9), but not detected in other samples; the contents of mecambridine (**5**) and albore (**7**) were low and varied among the various *Meconopsis* species. The variation in contents of the different alkaloids may be responsible for the different therapeutic efficacies of the herbal plants.

This study employed the BBDs to optimize the gradient mobile phase of UHPLC and ESI response of QTOF–MS for the rapid analysis of alkaloids in six *Meconopsis* species. Compared to the conventional LC–MS method, this optimized UHPLC–QTOF-MS method offered shorter analysis time and more sensitive detection of the alkaloids in plant matrices.

3.4. Identification of main alkaloids in Meconopsis species

The optimized UHPLC–QTOF-MS method was employed to analyze the components in the nine samples from six *Meconopsis* species. More than 20 peaks were detected from the crude extracts of the *Meconopsis* samples (as shown in Table 6 and Fig. 4). Trace amounts of the flavonoids were more polar than the alkaloids, with the peaks below 2.5 min analysis time corresponding to flavonoids, and the peaks above 2.5 min corresponding to alkaloids. By comparing the UV and high resolution ESI-MS/MS spectra data and retention time with the authentic compounds, eight of them were unequivocally identified, namely tricin (**1**), hydnocarpin (**2**), *O*-methylflavinantine (**3**), oleracein E (**4**), mecambridine (**5**), protopine (**6**), alborine (**7**) and berberine (**8**).The alkaloids showed abundant $[M+H]^+$ ion in the positive mode, and major fragments $[M+H-18]^+$, $[M+H-28]^+$ and $[M+H-16]^+$ corresponded to the loss of H_2O, CO and CH_4, respectively. Seven other peaks (peaks 9–18) could only be tentatively identified as 8,9-dihydroprooxocryptochine (**9**) [11], magnofloring (**10**) [38], 6-methoxy-17-methyl-2,3-[methylenebis(oxy)]-morphinan-

5-en-7-one (**11**) [39], reframoline (**12**) [11], mecambroline (**13**) [38], amurensine (**14**) [40], amurensinine (**15**) [38], meconoquintupline (**16**) [41], cryptopine (**17**) [38], and corysamine (**18**) [38], by comparing the HR-MS and MS/MS data with literature. In general, all nine samples of the six *Meconopsi* species contain compounds **6**, **7**, **8**, and **18**, with high contents of compounds **6** and **18**. Despite the similarity of the main peaks, some differences existed among these samples. For example, *M. racemosa* and *M. horridula* contain more alkaloids, while *M. torquata* and *M. integrifolia* contain less alkaloids. The difference between these samples is a result of their originating from different *Meconopsi* species.

4. Conclusions

The surface response methodology Box–Behnken designs were applied to optimize major parameters that influence the gradient of UHPLC and ESI sensitivity. Under optimal condition, the best UHPLC separation of the alkaloids in nine samples from six *Meconopsi* species was achieved within 14 min. The limit of detections (LODs) of this optimized QTOF-MS method was 0.5–0.1 ng/ml, which is a nearly 10-fold sensitivity improvement upon the previously published LC–MS methods. The results of these experiments are therefore important to the instrumental optimization of both UHPLC separation and ESI response for QTOF-MS.

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