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Comprehensive chemical analysis of Venenum Bufonis by using liquid chromatography/electrospray ionization tandem mass spectrometry

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ABSTRACT

A rapid, sensitive and versatile liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method was developed for the comprehensive analyses of the chemical constituents contained in the Chinese medicine-Venenum Bufonis (VB, Chan' Su in Chinese). LC analysis was carried out on an Agilent Eclipse plus C_{18} RRHD column (2.1 × 150 mm, 1.8 μm) with a linear gradient solvent system of water (0.1% formic acid) and acetonitrile (0.1% formic acid) as mobile phase. Detection and quantification were performed by multiple reaction monitoring (MRM) transitions via electrospray ionization (ESI) source operating in the positive ionization mode. Through "Molecular Feature Extraction" (MFE), more than 900 features were detected from VB extracts. Among them, a total of 97 components were identified using the Agilent METLIN accurate mass matching database (DB) established according to those reported in the literatures. Further more, 30 high quality matches were obtained by comparisons of their accurate mass and retention times (AMRT) with those imported out in the developed personal database (METLIN DB with AMRT). The characteristic fragmentation pathways were proposed for the tentative characterization of four representative types of bufadienolides in the present work. The targeted MS/MS experiment of the 30 major compounds was performed for their quantification and semi-quantification. And 7 of them were quantified over the assaying concentration range of 5.0–500 pg/μL. The lowest limit of detection and quantification of them were 0.25–0.50 and 1.25–0.25 pg/μL, respectively. The recoveries varied from 83 to 106% depending on the chemical types and different extraction solvents. The remained 23 bufosteroids were simultaneously semi-quantified using three representative standard compounds as their standard references, respectively.

1. Introduction

Venenum Bufonis (VB), dried white secretion of the auricular glands from either *Bufo bufo gargarizans* Cantor or *B. melanostictus* Schneider, has been widely used in clinic as a cardiotonic, diuretic, anohyne and antineoplastic agent both in China and other Asian countries [1,2]. Since the first report by Pelletier in 1817, extensive chemical studies on the cardiotonic constituents from VB have been carried out and demonstrated that VB contains cardiotonic steroids (bufosteroids), indoleamines, peptides, amino acids, fatty acids, polysaccharides, and sterols. It is known that the main components exhibiting cardiotonic activity are bufosteroids having bufadienolide structure, with a six-membered unsaturated lactone ring substituted at the 17β-position. The bufosteroids are divided into two groups according to the substituted forms at the 3 β-position of the steroid: bufogenin as an unconjugated steroid; and bufotoxin as a steroid conjugate such as suberoyl arginine esters, dicarboxylic acid hemiesters, and 3-sulfates. And the activity of the bufotoxin was suggested to be superior to that of the corresponding bufogenin [2–4]. Recently, in an effort to obtain more novel analogues of bufadienolides with increased cytotoxicity and water solubility, more than 40 biotransformed products with the hydroxylation position mainly at 1β, 5β, 7β, 12β and 16α have been obtained by several microbial transformation [5–9]. It has been reported that the bufosteroids resemble digitalis and related cardioactive glycosides of plant origin both in their chemical structures and their digitalis-like properties, such as cardiotonic, respiration excitation, and antineoplastic activities [3,10–12]. Recently, bufadienolides contained in VB have been widely reported to exhibit significant inhibitory activities against human myeloid leukemia cell lines (K562, U937, ML1, HL60), human hepatoma cell lines (SMMC7221), and prostate cancer cell line (LNCaP, DU105, PC3) [13–21]. Kamano et al. reported the bioactivities of 80 bufadienolides and discussed their structure–activity relationships on the inhibition of colchicines-resistant primary liver carcinoma PLC/PRF/5 cells [22]. The indoleamines contained in VB also have been reported to possess convulsion, hallucinogen and cytotoxicity activities [23,24].

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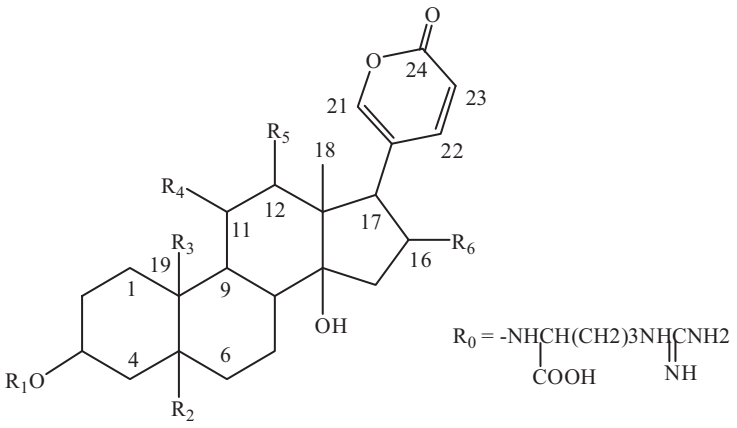

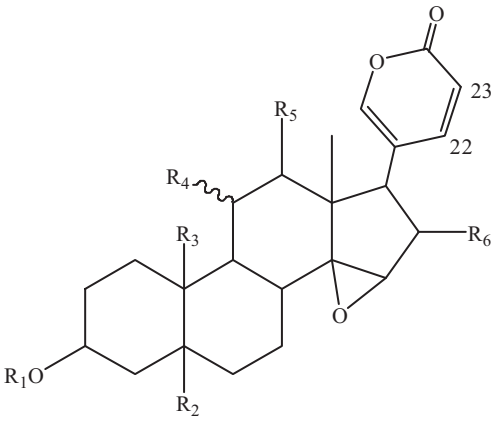

1. $R_1 = R_5 = R_6 = H$, $R_2 = R_4 = OH$, $R_3 = CH_3$, 9,11-ene

2. $R_1 = COCOOH$, $R_2 = R_5 = R_6 = H$, $R_3 = CH_3$, $R_4 = OH$

3. $R_1 = R_4 = R_5 = R_6 = H$, $R_5 = \alpha$-OH, $R_4 = (=O)$

4. $R_1 = R_4 = R_5 = R_6 = H$, $R_4 = \alpha$-OH, $R_3 = CH_3$

5. $R_1 = CO(CH_2)_6COOR_0$, $R_4 = \alpha$-OH, $R_3 = CH_3$, $R_4 = R_5 = R_6 = H$

6. $R_1 = R_2 = R_3 = R_6 = H$, $R_4 = \alpha$-OH, $R_5 = (=O)$

7. $R_1 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$, $R_2 = OH$

8. $R_1 = CO(CH_2)_6COOR_0$, $R_4 = R_5 = R_6 = H$, $R_3 = CH_3$, $R_2 = OH$

9. $R_1 = CO(CH_2)_2COOR_0$, $R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$

10. $R_1 = R_2 = R_5 = R_6 = H$, $R_4 = OH$, $R_3 = CH_3$

11. $R_1 = CO(CH_2)_7COOR_0$, $R_4 = \alpha$-OH, $R_3 = CH_3$, $R_4 = R_5 = R_6 = H$

12. $R_1 = R_4 = R_5 = H$, $R_2 = OH$, $R_3 = CHO$, $R_6 = COCH_3$

13. $R_1 = CO(CH_2)_3COOR_0$, $R_2 = R_4 = R_5 = H$, $R_3 = CH_3$, $R_6 = OCOCH_3$

14 $R_1 = R_4 = R_5 = R_6 = H$, $= CH_3$, $R_2 = OH$

15. $R_1 = CO(CH_2)_4COOR_0$, $R_2 = R_3 = R_6 = H$, $R_4 = \beta$-OH, $R_5 = (O)$, 9, 11-ene

16. $R_1 = CO(CH_2)_4COOR_0$, $R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$

17. $R_1 = R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_2OH$

18. $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CH_3$, $R_6 = OH$

19. $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CH_3$, $R_6 = OCOCH_3$

20. $R_1 = R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CHO$

21. $R_1 = R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$, 22(23)-epoxy

22. $R_1 = CO(CH_2)_5COOR_0$, $R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$, 4,5-ene

23. $R_1 = CO(CH_2)_8COOR_0$, $R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$

24. $R_1 = R_4 = R_5 = H$, $R_2 = OH$, $R_3 = CH_3$, $R_6 = OCOCH_3$, 22(23)-epoxy

25. $R_1 = R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$

26. $R_1 = CO(CH_2)_8COOR_0$, $R_2 = OH$, $R_3 = CHO$, $R_4 = R_5 = R_6 = H$

27. $R_1 = CO(CH_2)_6COOR_0$, $R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$, 4, 5-ene

28. $R_1 = R_2 = R_4 = R_5 = R_6 = H$, $R_3 = CH_3$

29. $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CH_3$, $R_6 = OCOCH_3$

30. $R_1 = CO(CH_2)_2COOR_0$, $R_2 = R_4 = R_5 = H$, $R_3 = CH_3$, $R_6 = OCOCH_3$

Fig. 1. Structures of 30 quantified and semi-quantified chemical constituents contained in Venenum Bufonis.

Considering the major bioactive compounds contained in VB, the bufasteroids are also known as the main toxic components contained in this medicine, and their toxic/therapeutic margins are narrow [10]. The quality control of VB is critical for its safety and effective usage in clinic. Several quality control methods have been carried out by using TLC, HPLC and GC analysis [25–29]. However, most of these investigations mainly focus on the analysis of the free-type hydrophobic bufadienolides. The whole profile of all the chemical constituents, especially the identification and quantification of the major bufotoxins contained in VB has not been reported yet. In our preliminary BIR bioactive screening experiment, the chloroform, methanol, ethanol and water extracts from VB all showed obvious cytotoxic activities against four human cancer cell lines (HepG2, H-1299, HT-29 and HCT-116, unpublished data). In the present work, a comprehensive description of the chemical constituents contained in different fractions of VB was carried out using a powerful UHPLC-UHD Q TOF MS/MS technique. Thirty compounds were chosen to carry out the targeted MS/MS analysis, and their structures were shown in Fig. 1. Through the METLIN accurate mass matching database analysis, yield 97 preliminary matches. Among them, 30 high quality matches were obtained through a further comparison of both their accurate mass and retention times with those in our personal database (AMRT database) established according to the literatures [1–3,27–29]. Their MRM chromatograms in four solvent extracts of VB were shown in Fig. 3.

2. Experimental

2.1. Materials and reagents

The medicine VB was purchased via Shenyang Pharmaceutical University from Pingyi Taifeng Medicine Materials IMP. & Exp.Co., Ltd. Shandong Province, China. The standard compounds, resi-

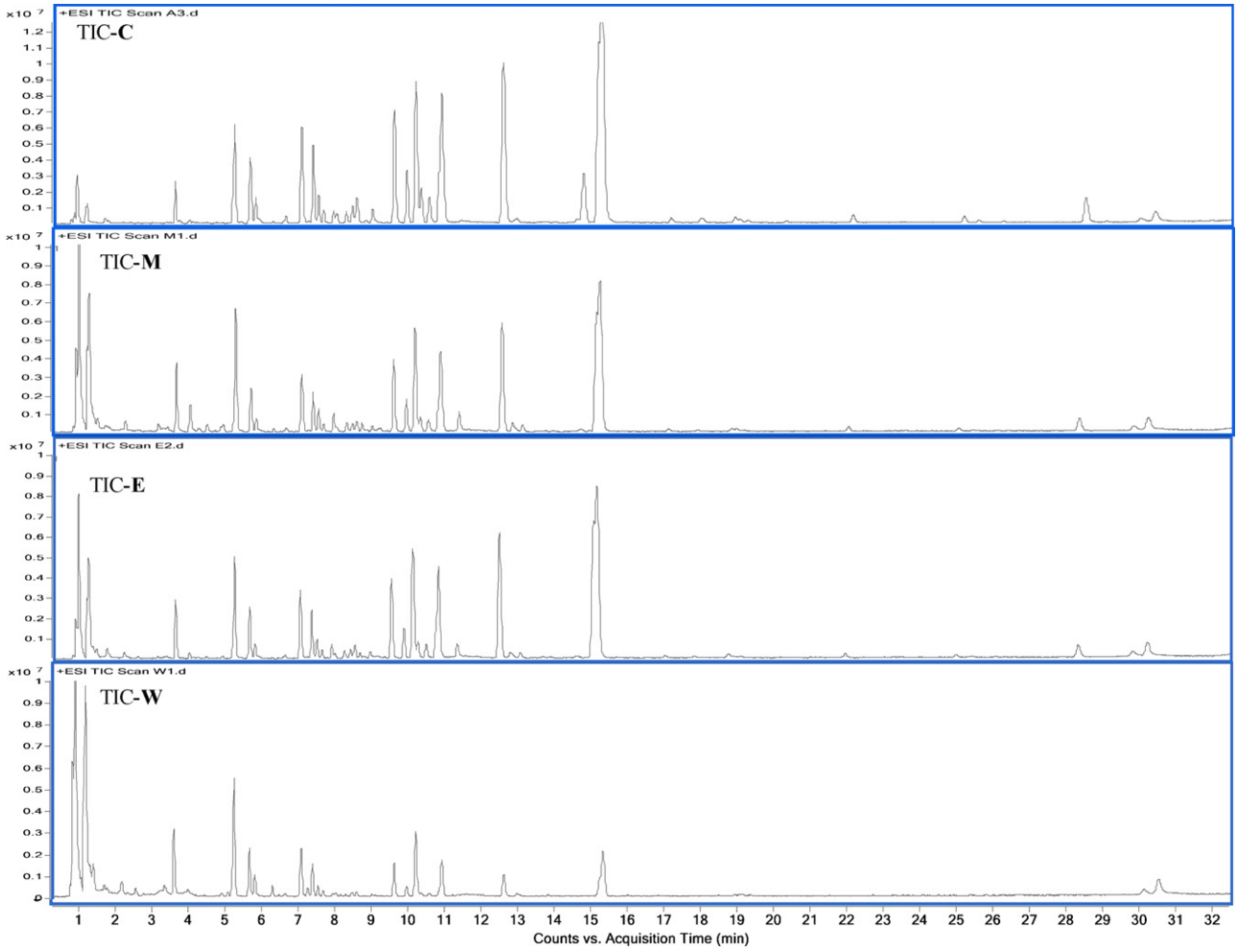

Fig. 2. Total ion chromatogram (TIC) of the chemical constituents contained in four solvent extracts from Venenum Bufonis. C: chloroform; M: methanol; E: ethanol; W: water.

bufogenin, arenobufagin gamabufotalin, bufalin, cinobufagin and bufotalin, were purchased from the China Institute for Control of Pharmaceutical and Biological products and the Hong Kong Jockey Club Chinese Medicine, respectively. The gamabufotalin-3-suberate-arginine was prepared and identified in our lab. The purity is above 97% through UV and LC/MS detection. Acetonitrile and formic acid are of HPLC grade (Sigma, USA). Ultra-pure water was prepared using a Milli Q-plus system (Billerica, MA, USA). All other reagents used for extraction are of analytical grade.

2.2. Sample preparation

The powdered VB sample (0.1 g) was exactly weighted and ultrasonic extracted with 10 mL chloroform for 1 h (1); 10 mL methanol for 1 h (2); 10 mL ethanol for 1 h (3); 10 mL ice-chilled Milli-Q water for 1 h (4). The methanol, ethanol and chloroform were recycled respectively and the residue was dissolved in 10 mL methanol as stock solutions. The water extract was dried through freeze-dehydration and re-dissolved in 10 mL 60% methanol as stock solution. All the stock solutions were diluted 1000 times with the mobile phase (40% B) and filtered through 0.22 μM membrane after centrifugation at 14,000 × *g* prior to LC/MS analysis.

2.3. Calibration curves, detection and quantification limits, and recovery

The seven standard compounds-resibufogenin, arenobufagin gamabufotalin, bufalin, cinobufagin, bufotalin and gamabufotalin-3-suberate-arginine were separately prepared in methanol (100 ng/μL) and stored at 4 °C as standard stock solutions. All the methanol stock solutions were stable during one month at 4 °C, and three months at −20 °C in the present experiment. The mixed standard solutions were prepared by mixing and diluting the above stock solutions in the mobile phase (40% B) to the final concentrations of 5, 10, 50, 100, 250 and 500 pg/μL. Calibration curves were calculated by the least squares linear regression method. The lowest limit of detection (LLOD) was calculated by using signal-to-noise (S/N) ratio of 3. The lowest limit of quantification (LLOQ) was determined by using S/N ratio of 10. Other 23 compounds were classified into three groups and semi-quantified using three types of their representative compounds as standard references respectively. To calculate the concentrations of any given analytes, their peak area ratios to three types of reference compound (gamabufotalin-3-suberate-arginine, cinobufagin or resibufogenin) were separately calculated and read off according to concentrations of the three known reference analytes. In detail, the un-conjugated bufadienolides (free type) were semi-quantified using cinobufagin as the standard reference, the conjugated bufadienolides (bufotoxins) were semi-quantified using the gamabufotalin-3-suberate-arginine as standard reference, while those bufadienolides with 16-acetyl group were semi-quantified using the cinobufagin as reference compound to calculate their peak area ratios and estimate their concentrations.

For the recovery calculation of the seven standard bufadienolides, the VB quality control (QC) samples (0.1 g) were extracted

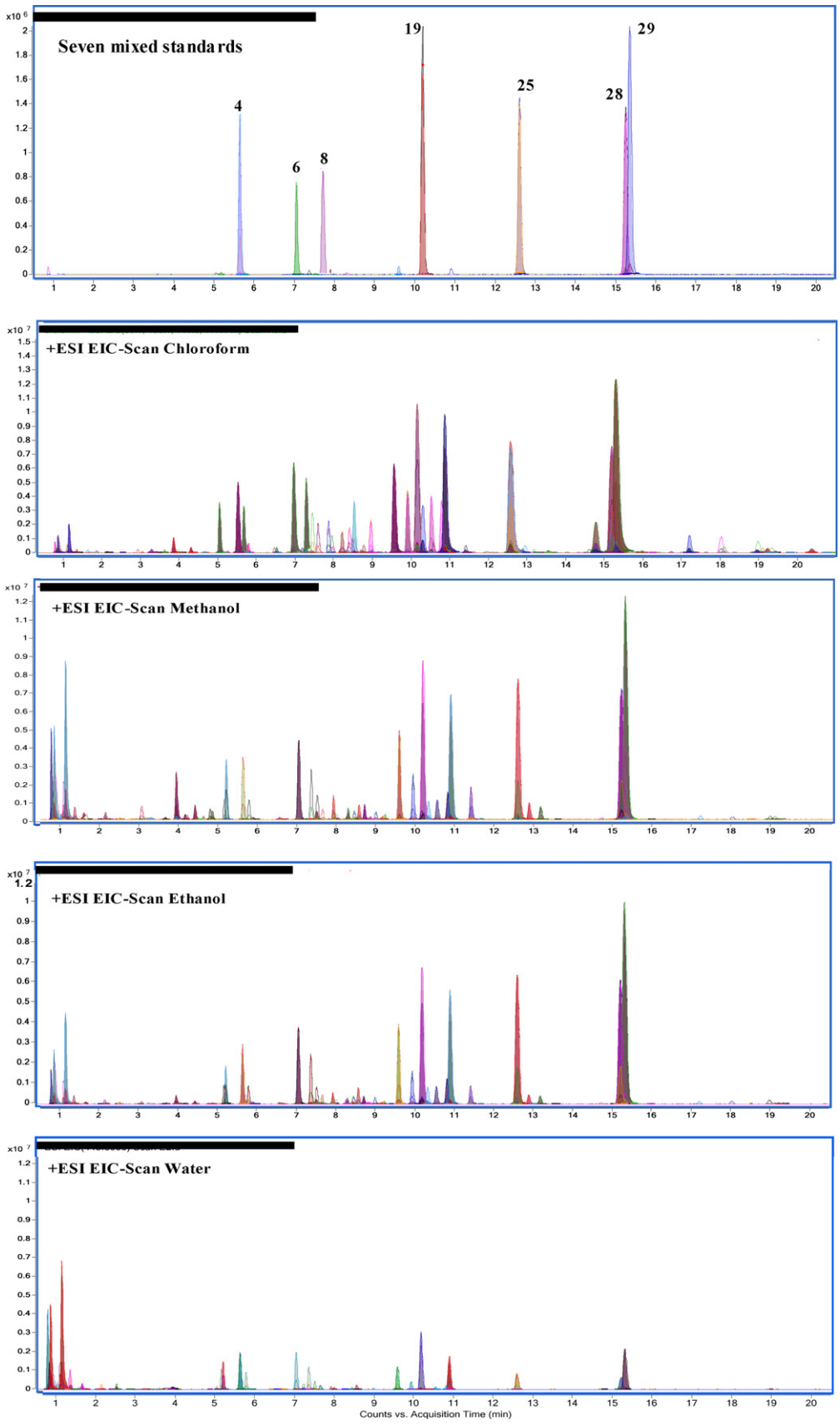

Fig. 3. The MRM chromatograms of seven standards and 30 quantified (semi-quantified) components in four solvent extract of Venenum Bufonis.

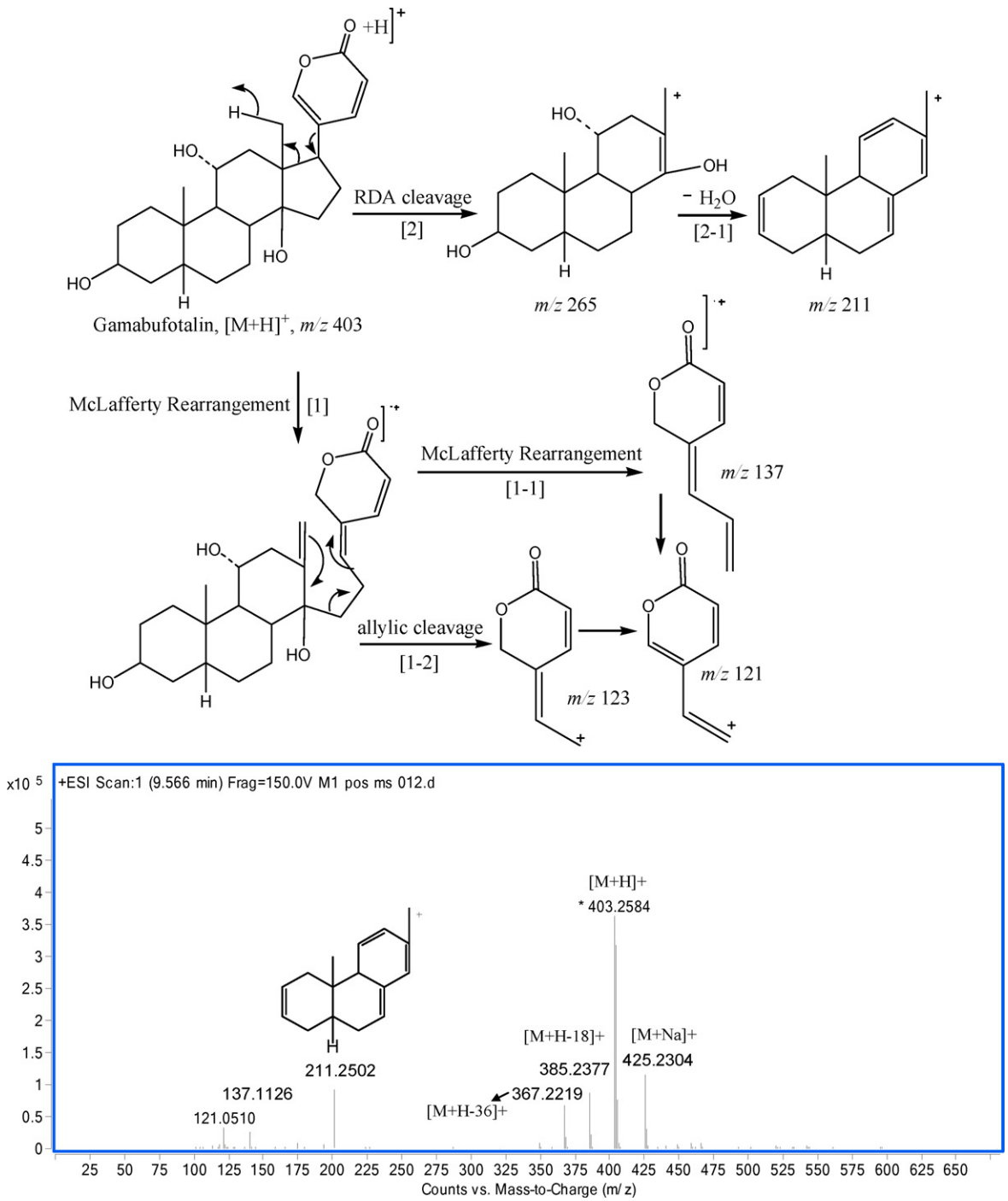

Fig. 4. Representative fragmentation pathway of cleavage type **I** and typical MS/MS spectrum of gamabufotalin.

with 10 mL of chloroform, methanol, ethanol and water, respectively. The mixed standard stock solutions were spiked into each extraction solvent (1.0 mL) at three concentration levels (5, 50, 500 pg/μL), the solvent was dried under nitrogen and reconstituted in 1.0 mL methanol (60% methanol for water extract) and then diluted 1000 times using mobile phase prior to LC/MS analysis. The concentrations of the QC samples before and after spiking of each analyte were calculated from the calibration curves, respectively, and the concentration ratios of founded/added analytes were used to estimate the recovery of each analyte in different extraction solvents. The resulting ratio value showed the recovery of each analyte expressed as percent of initial spiking concentration. Three replicates of VB samples at three concentration levels of each analyte were included in this recovery experiment of each extraction solvent. Overall, the recovery result indicates no noticeable interference resulting from different extraction solvents in the present experiment.

2.4. LC–MS/MS analysis

(**I**) Equipment: an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS mass spectrometer (Agilent Technologies) was connected to the UHPLC instrument (Agilent 1290 infinity) via an ESI ion source with JetStream technology for the comprehensive LC/MS/MS analysis of VB. An Agilent 6450 Triple Quadrupole LC/MS system accompanied with MassHunter Quantification Analysis software

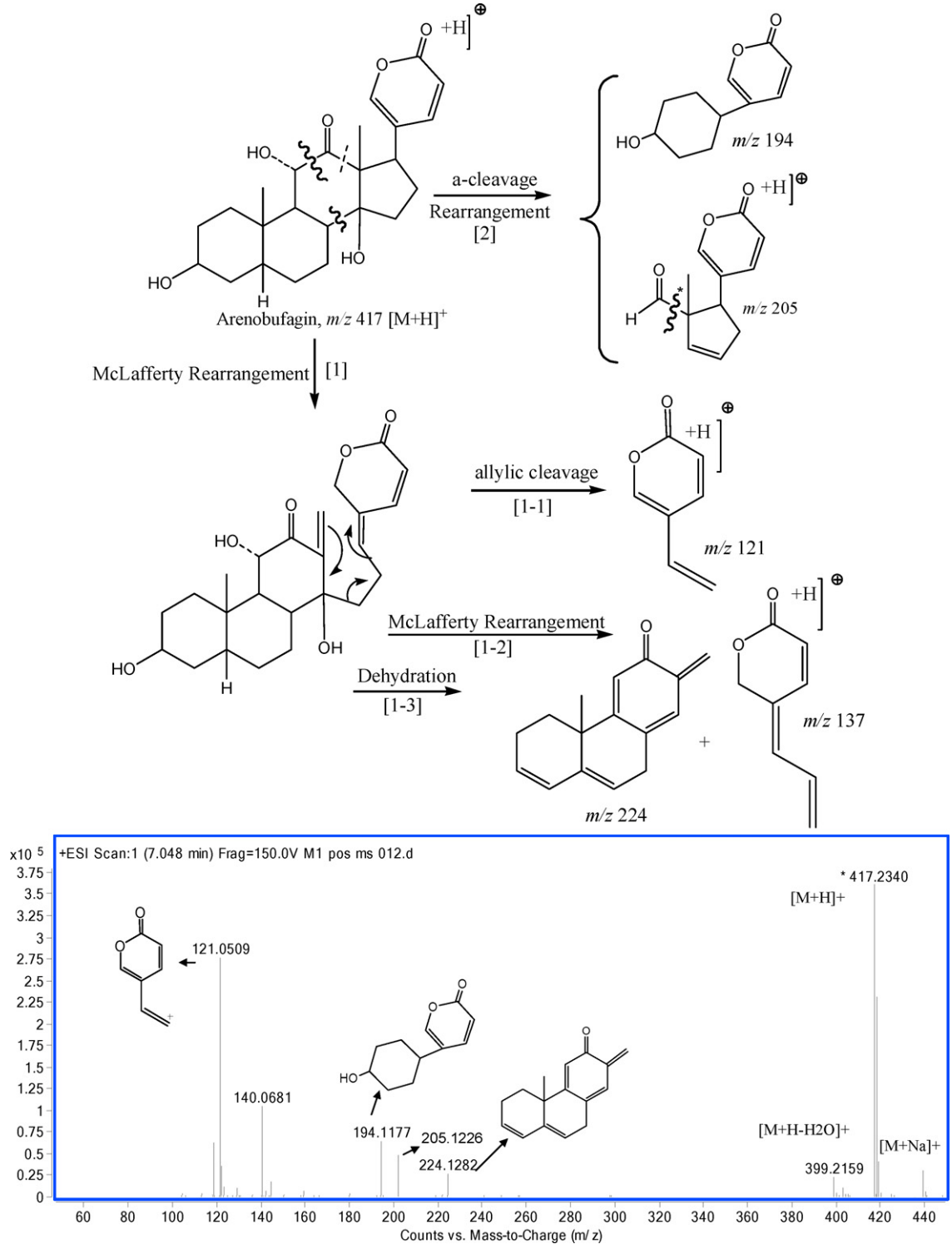

Fig. 5. Representative fragmentation pathway of cleavage type **II** and the typical MS/MS spectrum of arenobufagin.

was connected to the UHPLC instrument (Agilent 1290 infinity) for the specific quantification and semi-quantification of major bufosteroids. (**II**) LC conditions: the UHPLC conditions for LC–MS analysis were performed as following: an Agilent Eclipse plus C_{18} RRHD column (2.1 mm × 150 mm, 1.8 μm) was used at 40 °C. A gradient elution of solvent A (Milli-Q water contains 0.1% formic acid) and solvent B (acetonitrile contains 0.1% formic acid) was applied as follows: 0–8 min, 15–35% B; 8–30 min, 35–60% B; 30–33 min, 60–62.5% B; 33–34 min, 62.5–95% B; 34–39 min, 95% B. A pre-equilibration period of 6 min was used between individual runs. The flow rate was 0.4 mL/min, and the injection volume was 2 μL. (**III**). MS/MS conditions: the ESI-MS spectra were acquired in positive modes. Ultra-pure nitrogen (N_2) was used as the nebulizing and sheath gas. Product ion scanning experiments were conducted using ultra-high-purity N_2 as collision gas and the collision energy was optimized for each representative analyte to generate the most abundant production ions. The product ion spectra were further used to select the precursor-product ion pairs for the development of MRM assays. The ESI parameters were set as following: the capillary voltage is 4.5 kV. The flow rate and temperature of sheath

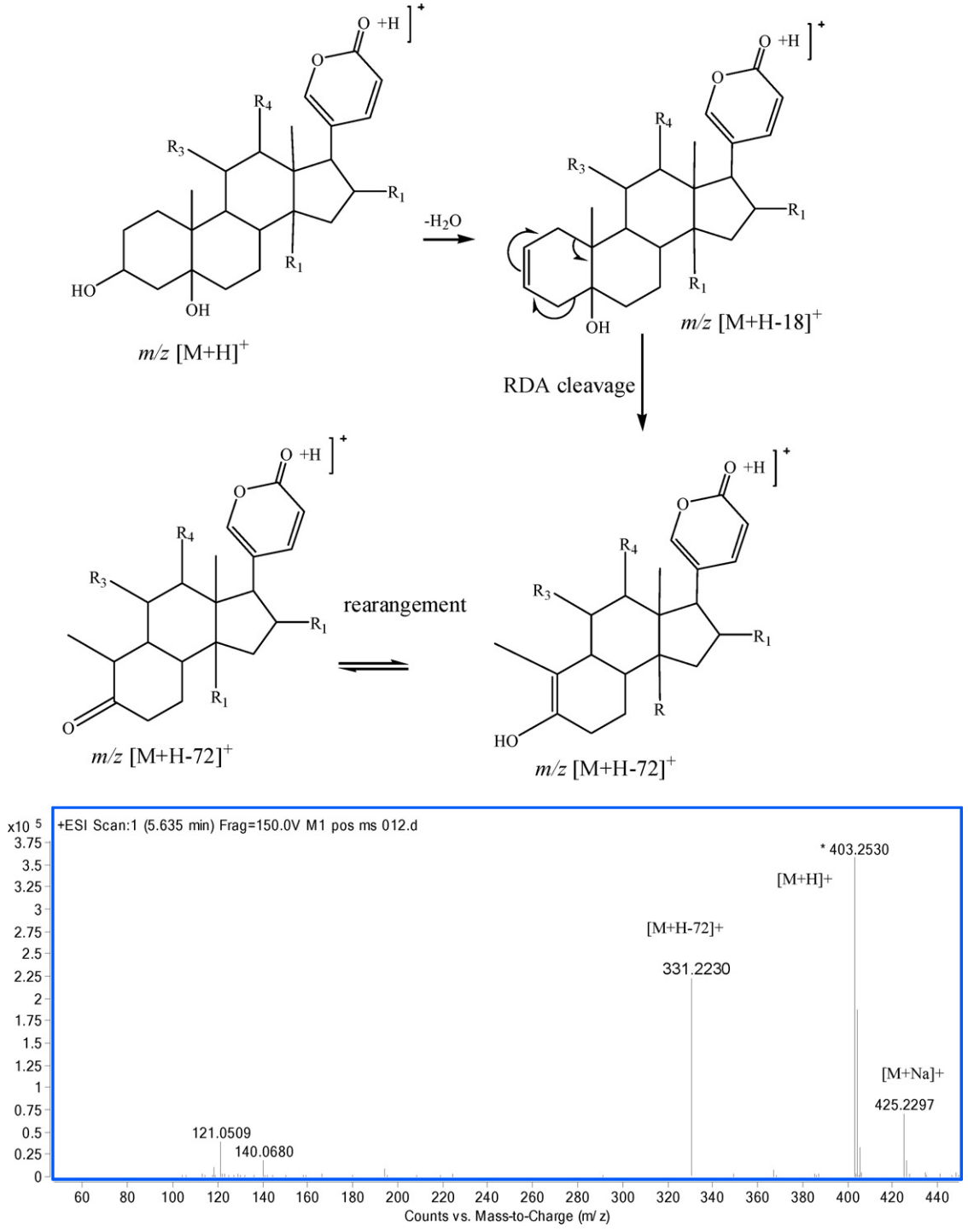

Fig. 6. Representative fragmentation pathway of cleavage type **III** and the typical MS/MS spectrum of telocinobufagin.

gas were 12 L/min and 400 °C, respectively. The flow rate and temperature of drying gas were 7 L/min and 300 °C, respectively. The pressure of nebulizer gas was 30 psi. The fragmentor voltage is 150 V. The mass analyzer was scanning from 100 to 2000 (*m*/*z*).

3. Results and discussion

3.1. Compounds identification and characterization from VB extract

The total ion chromatogram (TIC) of the chemical constituents contained in four solvent extracts from VB was shown in Fig. 2. In the present work, the mass fragmentation patterns were also elucidated to certificate the identification of these compounds. It showed that the unconjugated bufosteroids can produce a series of neutral ions or positive ions containing ring A, B and C following the continuous loss of neutral ion of H_2O (18) in the remained steroidal rings. At the same time, a series of fragmentation ions, such as $[M+H]^+$, $[M+H-61]^+$, $[M-H_2O-CO]^+$, $[M+H-COCH_3]^+$, and $[M+H-OCOCH_3]^+$ were obviously observed in this study. Four types of specific cleavage pathway were proposed for the bufosteroids identification (Figs. 4–7).

Cleavage type 1: The bufosteroids without 16-hydroxyl substitute, showed obvious ion peaks of lactonic-ring E at *m*/*z* 121

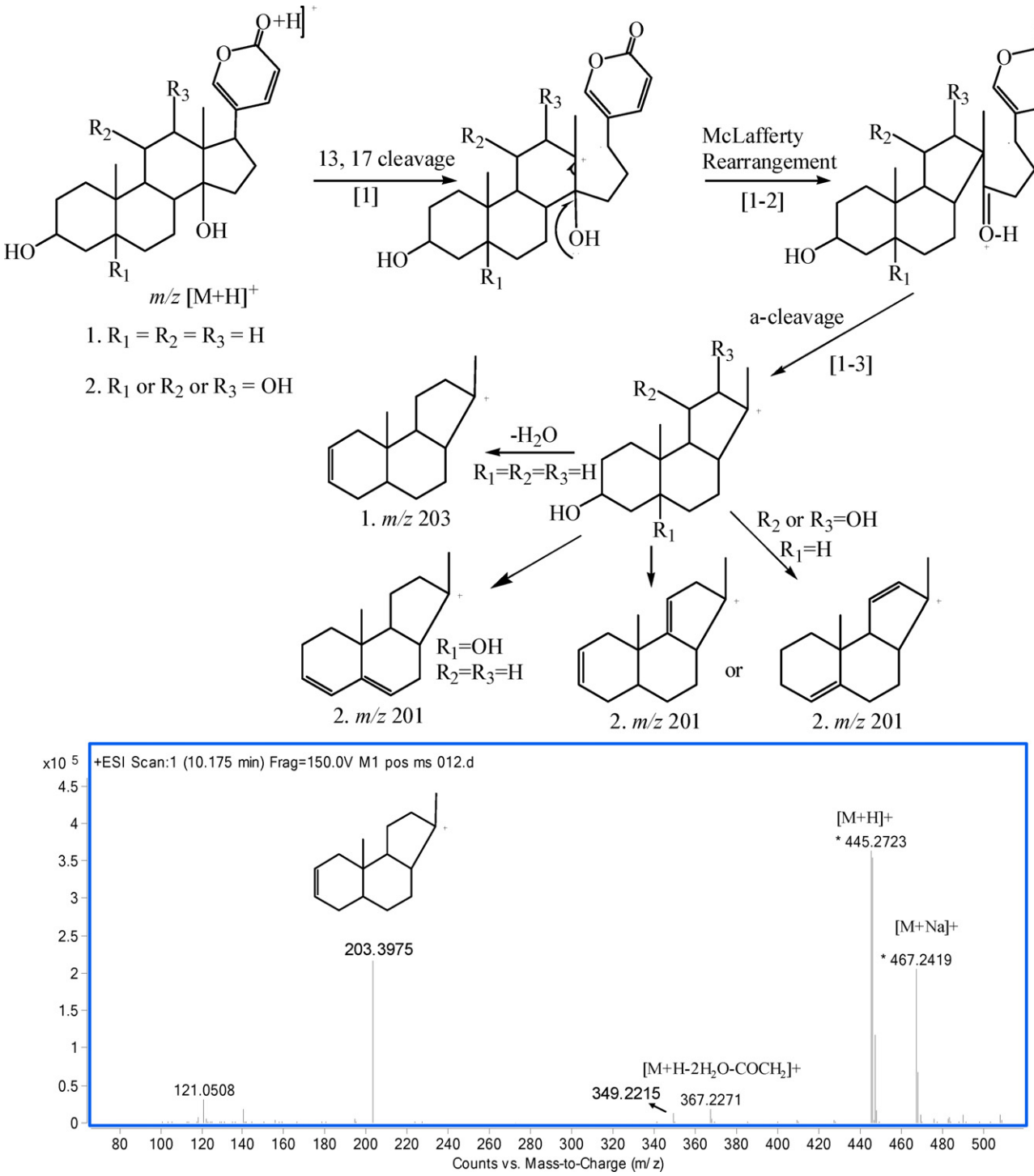

Fig. 7. The main fragmentation pathway of cleavage type **IV**, especially containing ring-A, B ions and typical MS/MS spectrum of compound bufotalin.

$[C_7H_5O_2]^+$ and *m/z* 137 $[C_8H_8O_2+H]^+$. The MS fragmentation mainly happened through a series of McLafferty Rearrangement cleavage and allylic cleavage between ring-D and ring-E (Fig. 4).

Cleavage type 2: Bufosteroids, such as arenobufagin, with 11-hydroxy and 12-oxo, can produce characteristic neutral ion signals of ring D/E at *m/z* 205, 194 through α-cleavage in ring C and dehydration in ring D (Fig. 5).

Cleavage type 3: Bufosteroids with 5-hydroxy in their structures showed the obvious ions at *m/z* $[M-H_2O+H]^+$, and $[M-72+H]^+$ through dehydration and RDA cleavage at ring A (Fig. 6).

Cleavage type 4: Bufosteroids with 14-hydroxy and another additional hydroxyl group (at C-3, C-5, C-11 or C-12 position) in the steroidal ring A, B or C showed dehydration cleavage ion of ring A, B, C at *m/z* $[C_{15}H_{23}]^+$ 203. While bufosteroid with 14-hydroxy and another two additional hydroxyl groups produced obvious ion at *m/z* $[C_{15}H_{21}]^+$ 201 (Fig. 7).

The MS results showed that a series of molecular ions of homologous compounds contained in VB simultaneously, which brings much difficulty for the exact differentiation from each other. To resolve this problem, the MS/MS values were obtained through MRM experiment, and the possible fragmentation pathway were

Table 1
Multiple reaction monitoring (MRM) transitions for the LC/ESI-MS/MS assay of 30 bufosteroids contained in Venenum Bufonis.

Comd. group	No.	Compounds	MRM (*m/z*) Positive mode	RT (min)	Molecular	Collision energy (eV)	Fragmentor voltage (V)
I	**28**	Resibufogenin[a]	385.2304 → 367.2500	15.171	$C_{24}H_{32}O_4$	20	150
I	**1**	Hellebrigenol-9,11ene	417.2200 → 399.2000	5.101	$C_{24}H_{32}O6$	20	150
I	**3**	φ-Bufarenogin	417.2207 → 399.2000	5.724	$C_{24}H_{32}O_6$	20	150
I	**4**	Gamabufotalin	403.2404 → 385.2000	5.847	$C_{24}H_{34}O5$	30	150
I	**6**	Arenobufagin	417.2100 → 399.2500	7.088	$C_{24}H_{32}O_6$	30	150
I	**7**	Telocinobufagin	403.2407 → 385.2000	7.612	$C_{24}H_{34}O_5$	30	150
I	**10**	11-Hydroxyl-resibufogenin	401.2251 → 383.2000	7.974	$C_{24}H_{32}O_5$	20	150
I	**14**	Marinobufagin	401.2252 → 365.2000	8.929	$C_{24}H_{32}O_5$	20	150
I	**17**	19-Hydroxyl-bufalin	403.2255 → 385.2500	9.532	$C_{24}H_{34}O_5$	20	150
I	**18**	De-acetyl-cinobufagin	401.2250 → 365.2000	9.862	$C_{24}H_{32}O_5$	20	150
I	**20**	Resibufagin	399.2100 → 381.2000	10.232	$C_{24}H_{30}O_5$	20	150
I	**21**	22,23-Epoxyresibufogenin	401.2255 → 365.2000	10.528	$C_{24}H_{32}O_5$	20	150
I	**24**	22,23-Epoxycinobufotalin	473.2460 → 417.2500	11.408	$C_{26}H_{32}O_8$	10	150
I	**25**	Bufalin	387.2500 → 369.2500	12.619	$C_{24}H_{34}O_4$	20	150
II	**8**	Gamabufotalin-3-suberate-arginine[a]	715.3825 → 697.4000	7.831	$C_{37}H_{54}N4O_{10}$	40	150
II	**2**	Gamabufotalin-3-oxalate	475.2197 → 415.2200	5.579	$C_{26}H_{34}O_8$	10	150
II	**5**	Telocinobufagin3-suberate-arginine	715.4192 → 331.2000	6.765	$C_{37}H_{54}N_4O_{10}$	40	150
II	**9**	Bufalin-3-succinate-arginine	643.3500 → 275.1500	7.937	$C_{34}H_{50}N_4O_8$	30	150
II	**11**	Gamabufotalin-3-pelargonate-arginine	729.3994 → 331.2000	8.235	$C_{39}H_{62}N_4O_9$	40	150
II	**13**	Bufotalin-3-glutarate-arginine	715.4189 → 697.4000	8.642	$C_{37}H_{54}N_4O_{10}$	40	150
II	**15**	Argentinogenin-3-adipate-arginine	699.3521 → 331.3500	9.116	$C_{38}H_{58}N_4O_8$	30	150
II	**16**	Bufalin-3-adipate-arginine	671.3931 → 303.1500	9.259	$C_{36}H_{54}N_4O_7$	30	150
II	**22**	Scillarenin-3-pimelate-arginine	683.3933 → 665.4000	10.867	$C_{37}H_{54}N_4O_8$	40	150
II	**23**	Bufalin-3-sebacate-arginine	727.4548 → 709.4500	10.967	$C_{39}H_{60}N_4O_9$	40	150
II	**26**	Bufotalinin-3-sebacate-arginine	755.4153 → 331.2000	12.918	$C_{40}H_{58}N_4O_{10}$	40	150
II	**27**	Scillarenin-3-suberate-arginine	697.4087 → 331.2500	13.209	$C_{38}H_{56}N_4O_8$	40	150
II	**30**	Cinobufagin-3-semi-succinate	543.2510 → 365.2000	19.002	$C_{32}H_{46}O_7$	30	150
III	**29**	Cinobufagin[a]	443.2356 → 365.2000	15.266	$C_{24}H_{34}O_6$	20	150
III	**12**	19-Oxo-cinobufotalin	473.2094 → 395.2000	8.488	$C_{26}H_{32}O_8$	30	150
III	**19**	Bufotalin	445.2512 → 349.2000	10.127	$C_{26}H_{36}O6$	20	150

[a] Standard compounds used as the reference standard of each group for semi-quantification of bufosteroids. Group **I**: free-type bufosteroids (bufogenin); **II**: 3-substituded conjugates of bufosteroids (bufotoxin); **III**: 16-acetyl-bufosteroids. RT: retention time.

proposed and compared between different isomers. The positions of hydroxyl and ketonic groups, which usually affect the polarities of these compounds, were also analyzed in the present work. Consequently, the accurate MS values, the proposed MS/MS fragmentation patterns together with their retention times were used for the tentatively characterization and identification of different homologous isomers. Six major isomerides (**A**, **B**, **C**, **D**, **E**, **F**) were detected at *m/z* 417 $[M+H]^+$ ($C_{24}H_{32}O_6$) at different retention times. Among them, isomerides **A**, **B**, **C** and **D** showed similar MS/MS fragmentation patterns with a series of characteristic MS/MS ion peaks at *m/z* 137 and 121 (Fig. 4). A characteristic neutral ion signal at *m/z* 194 was observed in compound **B** (arenobufagin) with the 11-hydroxy and 12-oxo (Fig. 5). Compound **B** was also unambiguously identified through the direct comparison with the standard arenobufagin. Compounds **D** (bufotalidin) with a 5-hydroxyl group showed ion signals at *m/z* 399 $[M-H_2O+H]^+$ and 345 $[M-72+H]^+$ through a series of de-hydration and RDA cleavage reactions (Fig. 6). Compounds **E** (de-acetyl-cinobufotalin) and **F** (de-acetyl-cinobufaginol) with 16-hydroxyl and 14, 15-epoxyl groups all showed characteristic molecular ions at *m/z* 401 $[M-16+H]^+$ and 399 $[M-H_2O+H]^+$. For the isomeride pairs **A**, **C**, and **E**, **F** with similar fragmentation pathway, the molecular polarities will be used to differentiate them from each other. The sequence of moiety polarities were proposed as: –OH > 19-CHO > 11 (12)-CO > 14, 15-epoxy, and 12-OH > 11-OH > 5-OH > 16-OH > 19-OH.

3.2. LC–MS/MS optimization for quantification

The Agilent 6450 Triple Quadrupole LC/MS system was successfully applied for the quantification and semi-quantification in this

Table 2
Linearity, recovery, limit of detection (LOD) and limit of quantification (LOQ) of LC/MS/MS assay for seven representative standard bufosteroids in Venenum Bufonis.

Compounds	Equation *X*: concentration pg//μL; *Y*: peak area	Recovery ± SD (%)						
		R^2	LLOD	LLOQ (pg//μL)	C	M	E	W
Resibufogenin	$Y = 8651.4X + 26{,}401$	0.99	0.25	1.00	96 ± 1	98 ± 4	94 ± 2	91 ± 2
Gamabufotalin	$Y = 6737.7X + 42{,}262$	0.99	0.25	1.25	97 ± 1	100 ± 4	102 ± 3	88 ± 2
Arenobufagin	$Y = 2973X + 38{,}182$	0.99	0.10	1.00	103 ± 3	101 ± 5	93 ± 6	89 ± 2
Bufalin	$Y = 7669.7X + 57{,}594$	0.99	0.50	1.25	98 ± 2	101 ± 4	103 ± 2	93 ± 2
Gamabufotalin-3-suberate-arginine	$Y = 7632.3X + 17{,}7333$	0.99	0.50	2.50	92 ± 4	92 ± 6	89 ± 4	85 ± 2
Cinobufagin	$Y = 12{,}633X + 105{,}063$	0.99	0.25	1.25	102 ± 3	97 ± 4	95 ± 2	88 ± 3
Bufotalin	$Y = 8825.1X + 89{,}062$	0.99	0.25	1.25	101 ± 1	98 ± 1	96 ± 4	94 ± 5

C: chloroform; M: methanol; E: ethanol; W: water.
LLOD: lowest limit of detection; LLOQ: lowest limit of quantification.

Table 3
Quantification and semi-quantification of 30 bufosteroids detected from Venenum Bufonis samples.

Group	Compounds	Retention time (min)	Content[a] (ng/mL)				Accurate MS
			C	M	E	W	
I	**Resibufogenin**[a]	15.171	462.46	281.93	265.25	25.97	384.2304
I	Hellebrigenol-9,11ene	5.131	114.76	47.84	43.40	35.50	416.1875
I	φ-Bufarenogin	5.724	165.60	79.47	81.94	48.43	416.2207
I	Gamabufotalin[a]	5.847	205.34	97.26	95.31	83.64	402.2404
I	Arenobufagin[a]	7.088	324.96	216.85	212.79	108.61	416.2192
I	Telocinobufagin	7.412	266.52	142.12	139.76	41.45	402.2407
I	12-Hydroxyl-resibufogenin	7.974	46.98	15.62	16.47	6.89	400.2251
I	Marinobufagin	8.929	79.88	26.12	25.70	8.94	400.2252
I	19-Hydroxyl-bufalin	9.532	272.33	140.73	137.80	53.22	402.225
I	De-acetyl-cinobufagin	9.962	96.88	54.19	52.65	16.36	400.2095
I	Resibufagin	10.232	66.32	28.15	27.93	11.24	398.2200
I	22, 23-Epoxyresibufogenin	10.528	78.55	31.19	32.53	8.94	400.2255
I	22, 23-Epoxycinobufotalin	11.408	28.36	23.59	21.05	0	472.2460
I	Bufalin[a]	12.619	328.21	286.28	272.08	45.79	386.2301
II	**Gamabufotalin-3-suberate-arginine**[a]	7.731	93.44	74.15	73.97	24.72	714.3825
II	Gamabufotalin-3-oxalate	5.579	10.29	9.88	9.97	15.34	474.2197
II	Telocinobufagin3-suberate-arginine	6.665	16.37	9.84	9.06	10.51	714.4192
II	Bufalin-3-succinate-arginine	7.937	21.32	16.58	15.93	8.76	642.3620
II	Gamabufotalin-3-pelargonate-arginine	8.235	25.96	9.03	8.12	3.15	728.3994
II	Bufotalin-3-glutarate-arginine	8.642	42.34	19.27	18.49	4.94	714.4189
II	Argentinogenin-3-adipate-arginine	9.116	17.23	20.06	19.79	2.61	698.3521
II	Bufalin-3-adipate-arginine	9.259	10.17	12.54	12.03	2.11	670.3931
II	Scillarenin-3-pimelate-arginine	10.867	44.91	36.17	38.72	6.92	682.3933
II	Bufalin-3-sebacate-arginine	10.967	109.73	66.12	65.35	21.24	726.4548
II	Bufotalinin-3-sebacate-arginine	12.918	34.59	19.77	17.31	0	696.4087
II	Scillarenin-3-suberate-arginine	13.209	16.72	18.20	16.79	0	754.4153
II	Cinobufagin-3-semi-succinate	19.002	46.81	23.01	22.97	0	542.2445
III	**Cinobufagin**[a]	15.266	472.01	301.81	293.19	66.69	442.2356
III	19-Oxo-cinobufotalin	8.488	72.39	49.58	49.16	47.31	472.2094
III	Bufotalin[a]	10.127	338.31	197.38	195.27	79.31	444.2512

C: chloroform; M: methanol; E: ethanol; W: water.
Group **I**: free-type bufosteroids; **II**: 3-substituted conjugates of bufosteroids; **III**: 16-acetyl-bufosteroids.
[a] Major compounds for quantification.

experiment. A total of 30 bufosteroids together with their conjugates were firstly characterized by MS^2 scan and the MS/MS product ions were used to ascertain their precursor ions and to select product ions for usage in MRM mode, respectively. To obtain the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energies and collision energies were optimized. The MRM ion pairs and corresponding optimal collision energies applied in this experiment are present in Table 1.

3.3. Method validation and quantification of major bufosteroids in different VB extract

External calibrations were typically constructed for seven analytes by the least-squares linear regression method. The results confirmed the assay was linear over the tested ranges (Table 2). The recovery of them in different extraction solvents was evaluated by spiking the mixed standard compounds into different VB extractions. The average recovery ranged from 84 to 106% depending the compound structures and different extraction solvents. The LLOD ranged from 0.1 to 0.5 pg/μL, and the LLOQ ranged from 1.0 to 2.5 pg/μL in the present study. In summary, the content assay for the seven compounds presented in this work has been performed well, showing good linearity, recovery and sensitivity (Table 2).

Simultaneously, other 23 major bufosteroids were quantitatively estimated using three types of representative standard compounds as their standard references, respectively (Table 3). It showed that although most of the chemical constituents contained in VB can be extracted by using methanol or ethanol, chloroform proved to be an ideal solvent for both bufogenin and bufotoxin extraction in this experiment.

4. Conclusions

The LC/ESI-MS/MS method together with our developed database of the major chemical constituents contained in VB can be applied for the comprehensive analyses of the bufosteroids and their conjugated derivatives contained in this medicine and its related pharmaceuticals. Those microbial transformation products of bufosteroids usually varied in their hydroxylation positions. The present described method with the proposed fragmentation pathway can be used for the elucidation of a series of biotransformation homologous isomers consequently. Furthermore, this rapid, sensitive and accurate analytical method paves the way for the pharmacokinetic study of VB.

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