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Short communication

Identification of metabolites of crude and processed Fructus Corni in rats by microdialysis sampling coupled with electrospray ionization linear quadrupole ion trap mass spectrometry

Gang Cao [a], Hao Cai [b], Yun Zhang [a], Xiaodong Cong [a], Chengrong Zhang [a], Baochang Cai [a,b,*]

[a] Research Center of TCM Processing Technology, Zhejiang Chinese Medical University, Hangzhou 310053, PR China
[b] Engineering Center of State Ministry of Education for Standardization of Chinese Medicine Processing, Nanjing University of Chinese Medicine, Nanjing 210029, PR China

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ABSTRACT

A microdialysis (MD) sampling coupled with electrospray ionization linear quadrupole ion trap mass spectrometry (LTQ-MS^n) method has been developed for rapid and sensitive analysis of rat microdialysate metabolite profile of Fructus Corni, a well-known Traditional Chinese Medicine (TCM). The purified samples were separated by a reversed-phase HPLC with C_{18} column under a gradient elution. Parent compounds and metabolites of crude and processed Fructus Corni of Jiu Zheng Pin (JZP, JZP is produced after steaming the crude drug pre-steeped in wine) were detected by the on-line MS^n detector in negative scan model. The identification of the metabolites and their structural elucidation were performed by comparing the changes in molecular mass and defining sites of biotransformation based on the accurate MS^n spectral information of diagnostic fragment ions. In this work, we used such strategies for the identification of the parent compounds and metabolites of crude and processed Fructus Corni in rats, and seven parent compounds and three new metabolites of Fructus Corni were found in rats for the first time. This study provides important structural information regarding to the metabolism of crude Fructus Corni and its JZP. Furthermore, this work also demonstrated the possibilities of using microdialysis sampling coupled with LC–MS^n approach for identification of bioactive compounds from TCM *in vivo*.

1. Introduction

Fructus Corni is derived from the dry ripe sarcocarp of *Cornus officinalis Sieb. et Zucc*. used for nourishing liver and kidney [1]. It has attracted increasing paid much attention as one of the most popular and cherish herbal medicine in clinic. It can be used for medicine, dietary supplement and cosmetic due to its biological and pharmacological activities such as anti-inflammation, anti-virus and anti-oxidation. Although pharmacological activities of crude Fructus Corni and its processed products have been extensively studied, very little is known about their *in vivo* characteristics. Previous studies only showed that morroniside and loganin were detected in rat plasma after i.g. administration of the water extract of Fructus Corni [2]. Unlike the single chemical compound, herbal medicines show extremely complicated pharmacological reactions. Constituents in herbal medicines may be substrates, inhibitors, leading to some synergetic and/or antagonistic reactions among different compounds [3]. One category of compounds contained in TCM attracting more public interests is the iridoid glycosides, which exist as prevalent groups of herbal medicines in nature, displaying a wide range of biological and pharmacological properties. Iridoid glycosides widely distributed in crude Fructus Corni and its processed products as the major active components [4,5]. Exploring dynamic of compounds in dialysate from Fructus Corni and its processed may help to explain why crude and processed Fructus Corni have traditionally been used for treating different clinical symptoms and expatiates upon the process principium [6]. This work could provide a scientific basis for elevating the quality of Chinese herbs and their compound preparations and optimizing the dosage regimens, as well as contributing the knowledges of searching active metabolites for drug discovery.

Despite the recent advancement of various analytical tools, the metabolite identification for the compounds undergoing multiple and unpredictable metabolism from the biological matrix remains a great challenge. In the past few years, mass spectrometry (MS) coupled with chromatographic separation has become a powerful and frequently used technique for metabolite identification [7,8]. Currently, subsequent innovations like the mass range extension, development of MS^n capabilities and improvement of high mass resolution make the ion trap mass spectrometers capable of analyzing biological macromolecules [9,10]. Today commercially

* Corresponding author at: Research Center of TCM Processing Technology, Zhejiang Chinese Medical University, Hangzhou 310053, PR China.
E-mail address: bccai@hotmail.com (B. Cai).

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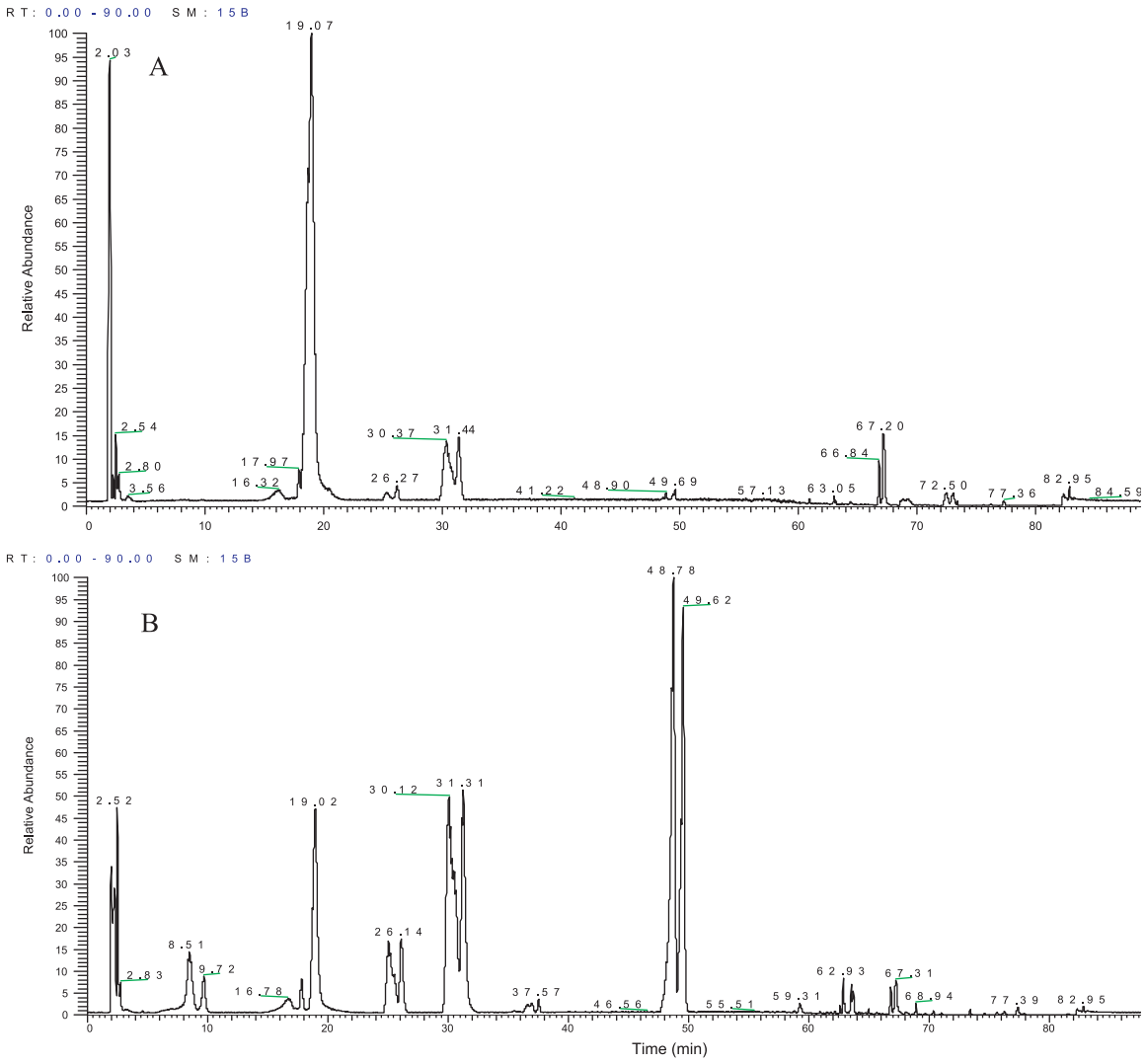

Fig. 1. LC/MS^n TIC chromatograms of microdialysis sample from Fructus Corni in negative mode. Blood dialysate sample 24 h after an i.g. administration of crude Fructus Corni extract (A); blood dialysate sample 24 h after an i.g. administration of processed Fructus Corni extract (B).

available ion trap mass spectrometers have many advantages, including the small size, low cost, high sensitivity and MS^n capability.

Traditional sample pretreatment methods include protein removal by precipitation with an acid or an organic solvent followed by centrifugation [11,12]. Most analytes require extraction from the supernatant into an organic phase after protein removal. Microdialysis is a powerful sampling technique based on passive diffusion of analyte across a semipermeable dialysis membrane with a defined certain molecular weight cut-off [13]. Since proteins are excluded from the sample by the dialysis membrane, microdialysis sampling seems to be an ideal approach for metabolism studies [14,15]. LC/MS and microdialysis for recovering an analyte continuously have been reported as a new analytical technique [16,17].

In this paper, based on microdialysis sampling combined with LC–MS^n, a fast and sensitive method for the identification of metabolites of crude Fructus Corni and its processed products in rat microdialysis samples was developed for the first time. The method allowed the detection of low-abundance metabolites along with their structural elucidation. Ten metabolites were confirmed, and three new metabolites were identified for the first time. This work has more comprehensively clarified the characteristics of crude Fructus Corni and its processed products, and has enriched our knowledge of *in vivo* metabolites of crude Fructus Corni and its processed products, which provides insight for further understanding mechanism of action of Fructus Corni. Therefore, this novel technique represents a particularly important way for evaluating the bioactivities of compounds in TCMs.

2. Experimental

2.1. Materials and reagents

The crude and processed forms of Fructus Corni of Jiu Zheng Pin (JZP) were collected from Henan suppliers. Acetonitrile was purchased from E. Merck (Darmstadt, Germany). Deionized water was purified using the Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid, sodium citrate, dextrose, and sodium chloride were obtained from E. Merck (Darmstadt, Germany). Solvents (Millipore Corp., Bedford, MA) of HPLC grade were used for all preparations. All other chemicals were of analytical grade and commercially available.

2.2. Animals

Fifteen male adult Sprague-Dawley rats weighing approximately 320 g were obtained from the Laboratory Animal Center

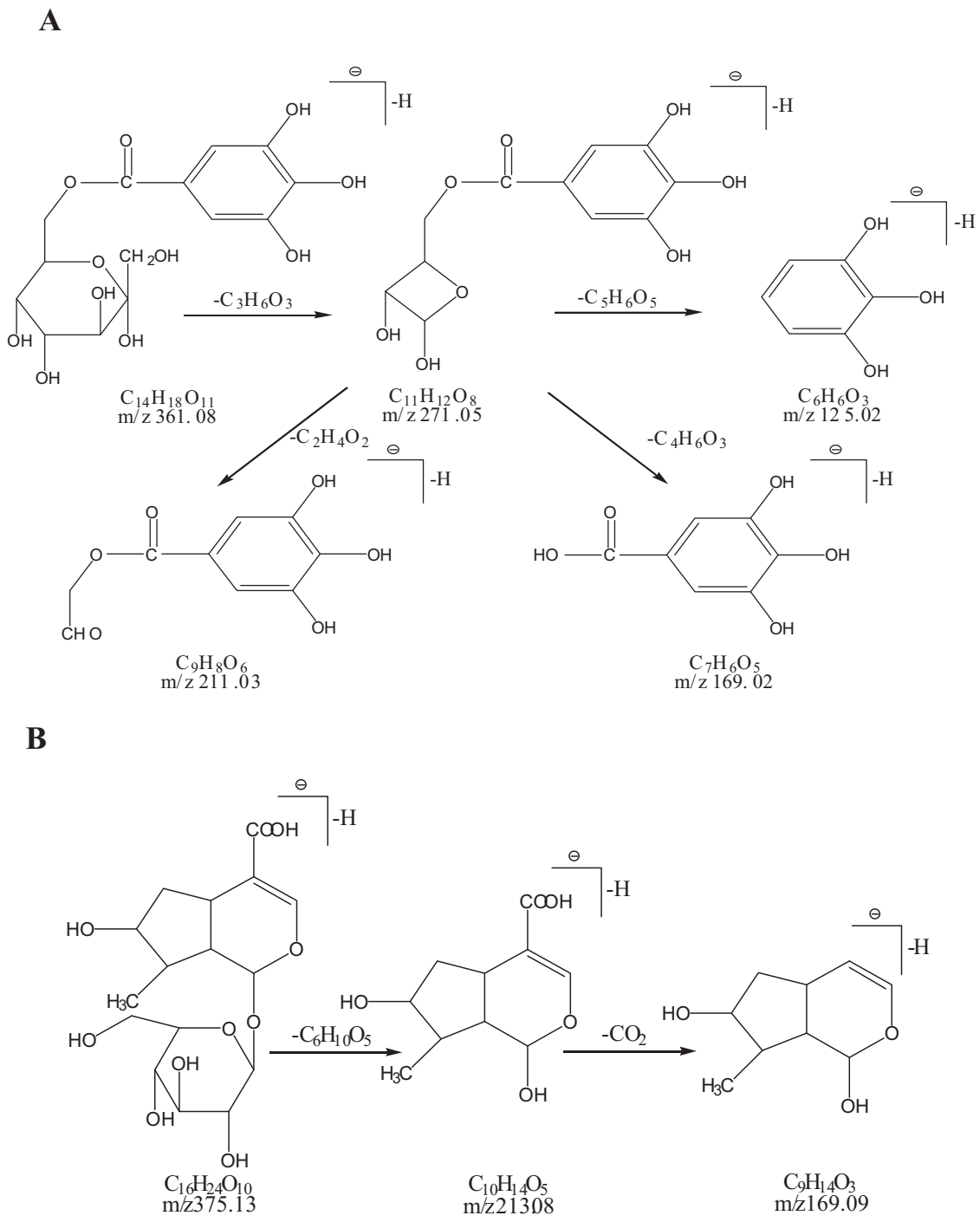

Fig. 2. The proposed MS^3 fragmentation pathways and the structures of 7-O-galloyl-D-sedoheptulose (A) and loganin acid (B) resulting from collisional activation.

of Zhejiang Academy of Medical Sciences (Zhejiang, China). Animals were acclimatized for at least 5 days with alternating 12 h dark/light cycles in a climate controlled room with the temperature maintained at 22 ± 1 °C and a relative humidity of 60 ± 10%. Water and standard laboratory food were available *ad libitum*. All experiments were performed according to the guidelines for the care and use of animals as established by Zhe Jiang University.

2.3. Preparation of sample solutions

100 g powder of Fructus Corni and its JZP samples were soaked together in 200 ml of water for 2 h at room temperature and thereafter refluxed for 2 h, respectively. The filtrate was collected and the residues were then refluxed twice in 1000 ml of water for 1.5 h. The three filtrates were combined and evaporated to the final volume of 100 ml under reduced pressure at a temperature not exceeding 60 °C.

2.4. Preparation of ACD solution

Anticoagulant citrate dextrose (ACD) solution consisting of 0.67 g sodium citrate, 0.24 g citric acid, and 1.50 g glucose, adjusted to pH 7.4, was used as the perfusion medium for the microdialysis probes.

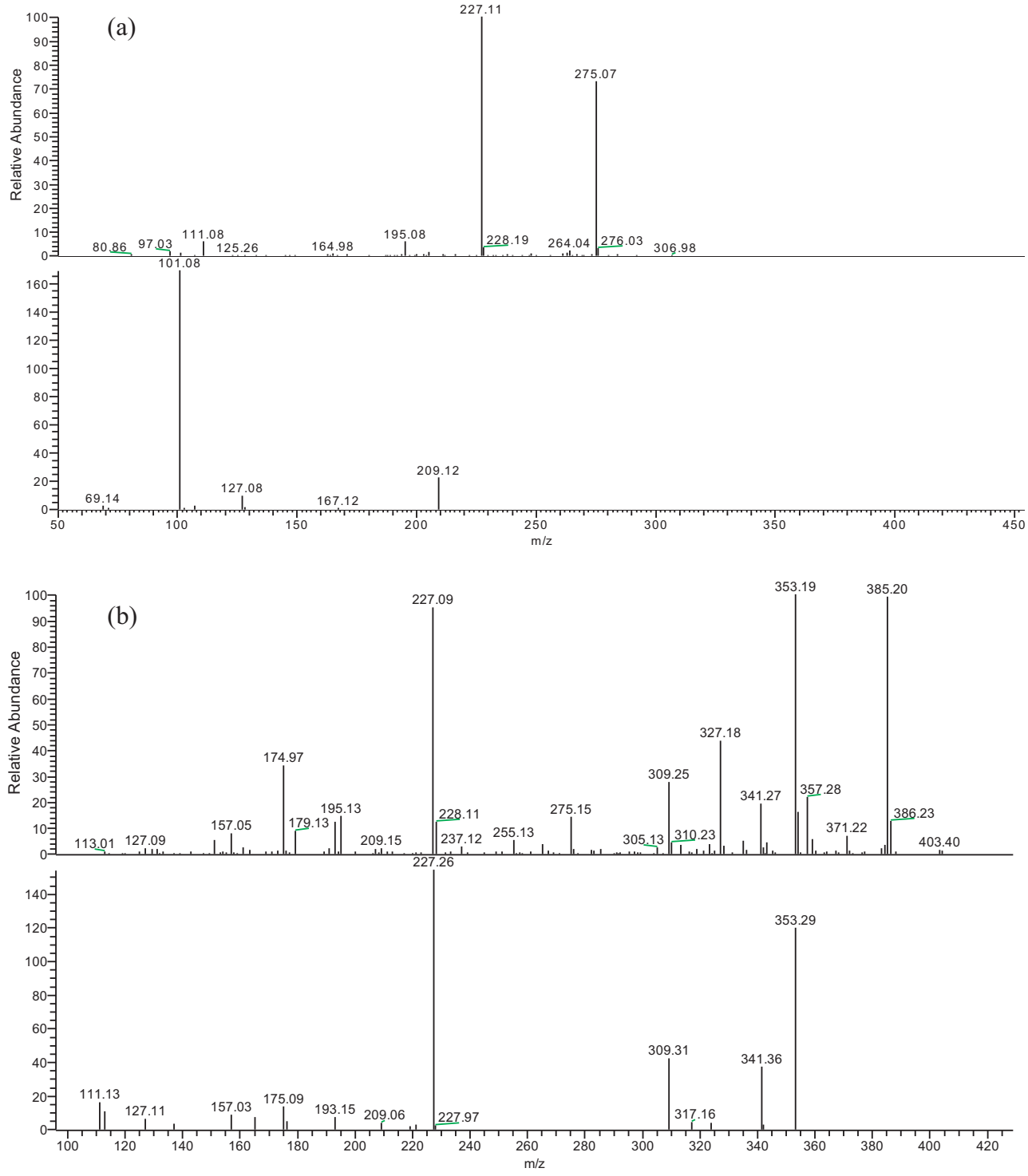

Fig. 3. Accurate MS 2 and MS 3 spectra of metabolites. (a) M1; (b) M2; (c) M3.

2.5. Surgical procedures

Sprague-Dawley rats were anaesthetized with ketamine/xylazine (90 ± 10 mg/kg) by intraperitoneal injection and mounted on a stereotaxic frame (Bioanalytical Systems, West Lafayette, IN, USA). A flexible vascular microdialysis probe (MD-2310, BAS, West Lafayette, IN, USA) with an O.D. of 0.5 mm, a membrane length of 10 mm and a nominal 18 kDa MW cut-off was inserted into the right jugular vein of pentobarbital-anaesthetized rats towards the right atrium, and perfused with perfusion fluid. A flexible wire mesh on the probe was sutured to the pectoral muscle. Inlet and outlet lines to the probe were housed within a single piece of flexible tubing, which was externalized via a surgical introducer needle. After surgery, the rats was allowed to recover for 2 days in single-animal cages under standard conditions (12 h light/dark cycle, a controlled ambient temperature of 22 ± 2 °C and a relative humidity of 60 ± 10%), with free access to food and water.

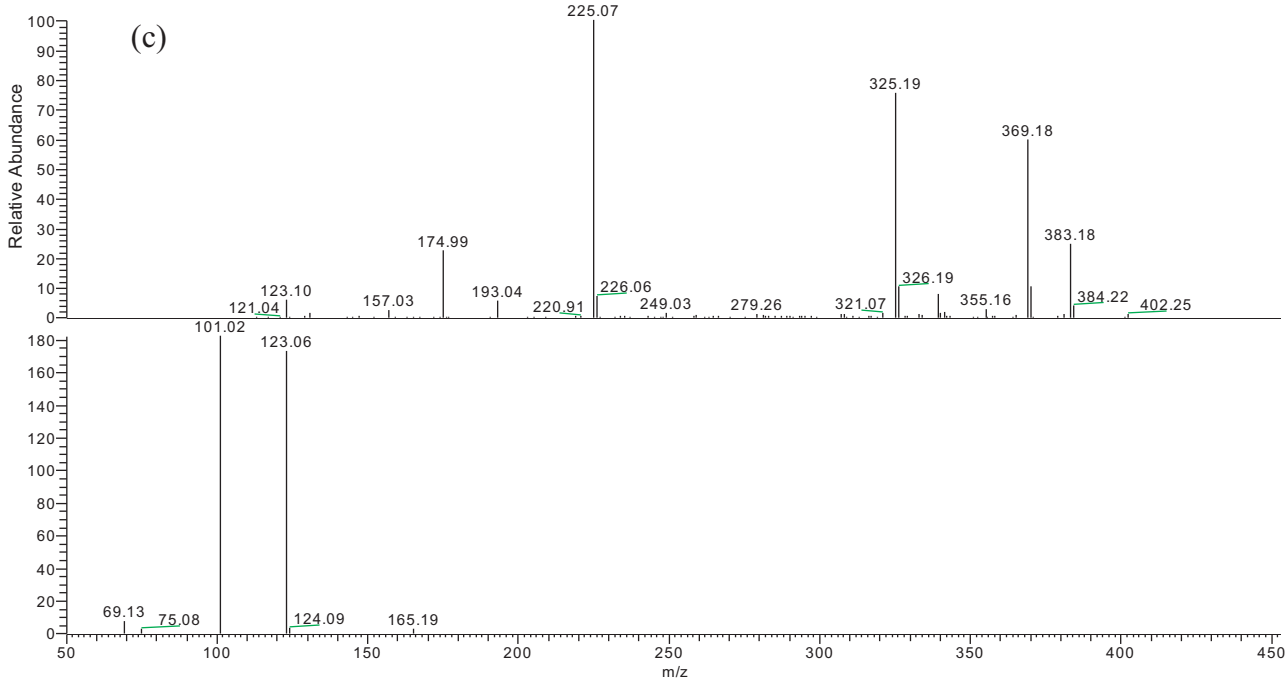

Fig. 3. (*Continued*).

2.6. In vivo microdialysis experiment

Fifteen rats were randomly divided into three groups (5 per group). After fasting the rats overnight, one group of animals while conscious was given the aqueous extract of crude Fructus Corni intragastrically at a dose of 2.0 ml/kg, while the other group was given the aqueous extract of JZP. A microdialysis probe (MD-2310) was inserted into the left jugular vein. The inlet of the probe was attached to a BAS syringe driver (MD-2310, USA) connecting to a controller (240 V/50 Hz MD-1000 K, BAS, West Lafayette, IN, USA), and filled with ACD solution as the perfusion fluid. The vascular microdialysis probe was continuously perfused at a flow rate of 2.0 μl/min. Following this equilibrium period, microdialysate was collected into 150 μl vials throughout the experiments using a refrigerated fraction collector (MD-1201, BAS, West Lafayette, IN, USA). All microdialysis experiments were performed with the freely moving animals being maintained in an awake animal caging system (Stand-Alone Raturn and Rodent Bowl Kit, BAS, West Lafayette, IN, USA), with no anaesthesia being given throughout the experiment. Microdialysate samples from the blood vessels were collected for an additional 300 min automatically and stored at −20 °C for centralized LC–MS^n analysis.

At the end of each washout period, blank dialysate samples were also collected to ensure that no drug was detected prior to its subsequent administration.

2.7. Chromatography

Analyses were performed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a diode array detector. The monitoring wavelength was set at 218 nm for gallic acid. An Agilent Zorbax Extend C18 column (250 mm × 4.6 mm, 5 μm) was used with a flow rate of 1.0 ml/min. The mobile phase consisted of 2% acetonitrile and 98% aqueous formic acid (0.1%, v/v) using a gradient elution of 2% B at 0–10 min, 2–5% B at 10–15 min, 5–15% B at 15–45 min, 15–25% B at 45–55 min, 25–90% B at 55–70 min, 90% B at 80 min. All samples were centrifuged at 15,000 × *g* for 10 min. The column temperature was maintained at 30 °C, and 2 μl aliquots of microdialysis sample were injected into the LC/ESI-MS^n system for analysis.

2.8. Mass spectrometry

All mass spectra were determined on a LTQ XL^{TM} linear ion trap instrument (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ion source, which is capable of analyzing ions up to *m/z* 2000. The scan scope was chosen from *m/z* 50 to 1000. By means of the comparison of ESI-MS^n spectra in positive ion mode and negative ion mode, the latter was chosen [18]. The spray voltage was set to −3.0 kV. The capillary voltage was fixed at 4.0 V and the temperature at 270 °C. The ion gauge pressure was 4.0×10^{-3} Pa. Nitrogen was used as a sheath gas and the flow rate was 40 arbitrary units. Helium was used as the buffer gas. Data acquisition and processing was performed using Xcalibur software (version 2.0.7, Thermo Fisher Scientific, Inc.) and Metworks (version 1.1.0).

3. Results and discussion

3.1. LC–MS^n analysis of the parent compounds and metabolites

To obtain a global view of rat microdialysate metabolite profiles, drug-containing microdialysate samples collected up to 24 h after oral administration of Fructus Corni preparation, was analyzed by LC/ESI-MS^n in negative ion modes. Fig. 1(a) and (b) showed the typical total ion chromatograms (TICs) of crude Fructus Corni-containing microdialysate samples and its JZP-containing microdialysate samples in negative ion mode, respectively. Besides seven parent compounds (P1–P7), there were three new peaks which could be attributed to metabolites in crude Fructus Corni-containing microdialysate and the JZP-containing microdialysate samples. As shown in Table 1, most of the parent compounds were identified by comparison of their retention times and MS^n model with the standards. Furthermore, the 7-O-galloyl-D-sedoheptulose and loganic acid were first found in rat microdialysate samples after i.g. administration of an aqueous extract of crude Fructus Corni and

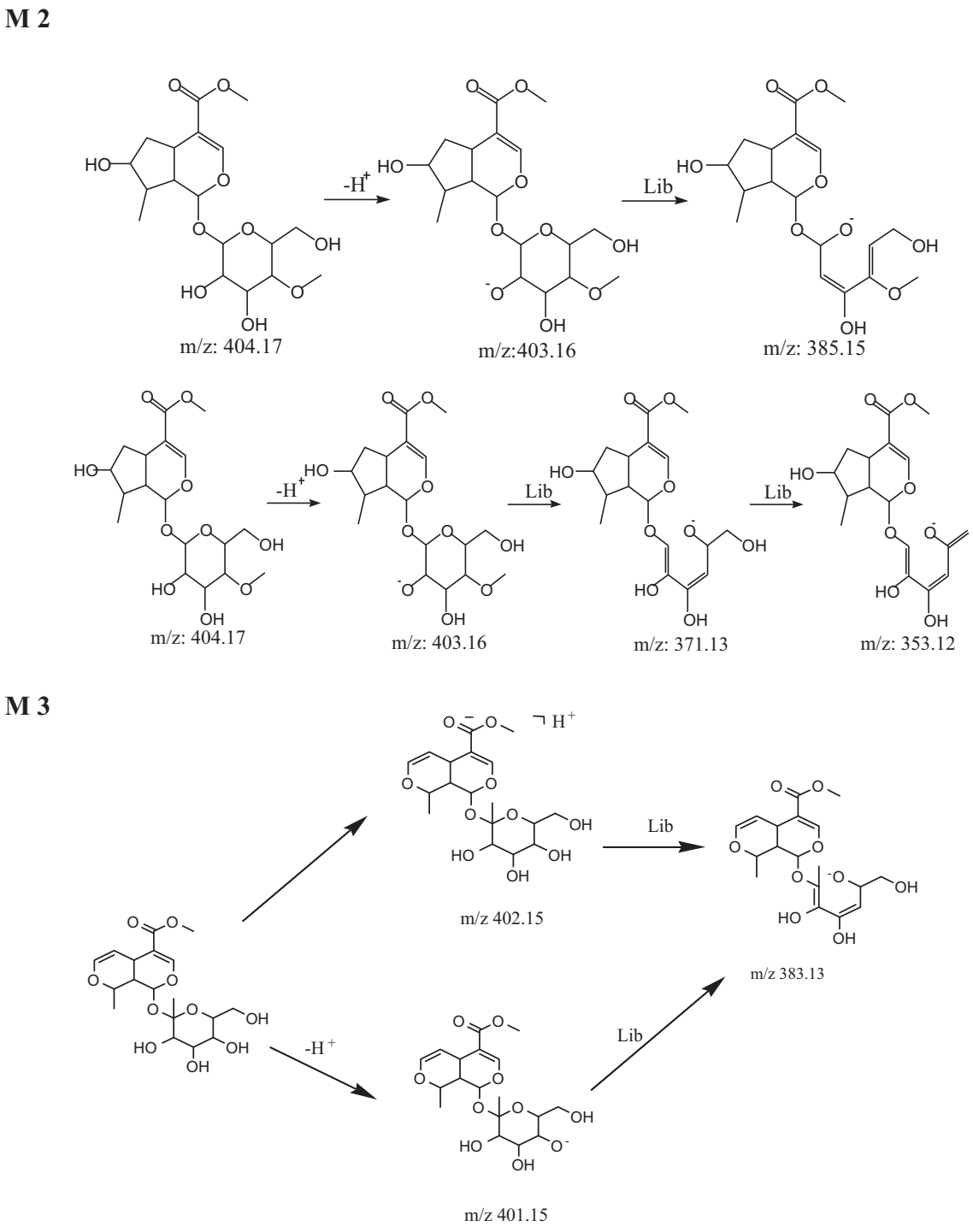

Fig. 4. The proposed MS3 fragmentation pathway and the structure of M2 and M3.

Table 1
The mass data of metabolites and parent components acquired using MD-LC-LTQ XL linear MS^n.

No.	Compound name	MW	RT (min)	Ion type	Parent ion	Product ion	Intensity (crude)	Intensity (processed)
1	Loganin acid	376	25.3	$[M-H]^-$	*m/z* 375	213, 169, 151, 95	2.99 E3	3.20 E3
2	Morroniside	406	25.8	$[M+HCOOH-H]^-$	*m/z* 451	405, 243, 141	2.53 E3	1.68 E4
3	7-O-ethylmorroniside	388	34.8	$[M-H]^-$	*m/z* 433	387, 225, 123	1.65 E3	2.29 E3
4	Sweroside	358	35.1	$[M-H]^-$	*m/z* 403	357, 195, 125, 81	1.44 E3	6.13 E3
5	Loganin	390	35.6	$[M+HCOOH-H]^-$	*m/z* 435	389, 227, 101	3.22 E3	3.81 E4
6	7-O-methylloganin	404	36.5	$[M-H]^-$	*m/z* 403	389, 227, 101	1.40 E3	8.23 E3
7	7-O-galloyl-D-sedoheptulose	362	40.5	$[M-H]^-$	*m/z* 361	361, 271, 211	1.20 E3	1.87 E3
8	M1	308	34.0	$[M-H]^-$	*m/z* 307	227, 101	1.50 E3	9.01 E3
9	M2	404	37.5	$[M-H]^-$	*m/z* 403	385, 363, 227	2.53 E3	1.14 E4
10	M3	402	37.6	$[M-H]^-$	*m/z* 401	369, 225	2.14 E3	3.97 E3

its JZP. The proposed MS^3 fragmentation Pathways of 7-O-galloyl-D-sedoheptulose and loganic acid were shown in Fig. 2.

3.2. Identification of iridoid glycoside-related metabolites

Iridoid glycosides are main constituents in crude and processed Fructus Corni. The three new compounds detected in microdialysate samples were tentatively assigned as metabolites originating from Iridoid glycosides. The accurate ion trap mass spectrum of metabolites in rat microdialysate was shown in Fig. 3. The intensities of metabolites were different in rat after i.g. administration of an aqueous extract of crude Fructus Corni and its JZP. The results were shown in Table 1.

3.3. Metabolite of M1

Metabolite M1 had a retention time of 34.0 min and showed the $[M-H]^-$ ion at m/z 307. In the mass spectrum of the metabolite M2, the spectra of the MS^3 mass spectrum m/z 307 > m/z 227 > can be observed the same with that of the MS^3 mass spectrum m/z 435 > m/z 227 > of loganin, demonstrating that M2 and A share the m/z 227 (loganin, by removal of glucose, can get m/z 227) structure part. From the MS/MS spectrum of the metabolite M2, it can be known that the molecular ion peak of M2 is m/z 307, differing 80 from m/z 227, thus it can be inferred that Phase II metabolism sulfonation (+SO_3, 80) might have occurred in the body; therefore, it can be obtained that sulfonation had occurred on the m/z 227 structure and the m/z 227 structure should have been obtained by removal of glucose from loganin. It can be further inferred that two metabolism processes as deglucuronidation (−162) and sulfonation (+80) had occurred on loganin.

3.4. Metabolite of M2

Metabolite M2 was eluted at retention 37.5 min. M2 appeared as main metabolites in rat microdialysate samples with $[M-H]^-$ ion at m/z 403, which could lead to two main MS^2 product ions at m/z 385 and 227. On the basis of the elemental compositions of fragment ions and bond connectivities present in the parent molecule, the most likely methylating position was located at the glycoside group. The speculated structure and the proposed MS^3 fragmentation pathway of M2 are shown in Fig. 4.

3.5. Metabolite of M3

Metabolite M3 had a retention time of 37.6 min and showed the $[M-H]^-$ ion at m/z 401. The molecular ion of M3 could lead to two main MS^2 ions at m/z 369 and 225 .In the mass spectrum of the metabolite M3, the spectra of the MS^3 mass spectrum m/z 401 > m/z 225 > can be observed the same with that of the MS^3 mass spectrum m/z 435 > m/z 227 > of 7-O-ethylmorroniside, suggesting that M3 and 7-O-ethylmorroniside share the m/z 225 (7-O-ethylmorroniside, by removal of glucose, can get m/z 225) structure part. These results indicated that M3 was methylated product and the methylated position should be located on the glycoside group. The structure and the proposed MS^3 fragmentation pathway were concluded and shown in Fig. 4.

4. Conclusion

In the present study, a method of microdialysis sampling coupled with electrospray ionization-LTQ XL linear ion trap mass spectrometry was developed to analyze rat microdialysate metabolites after i.g. administration of an aqueous extract of crude Fructus Corni and its JZP. The MS^n data with high mass accuracy provided much formation to investigate the structures of metabolites under electrospray ionization. The experimental results indicated that the absorption of crude Fructus Corni in rat was better than that of its JZP. Our study has demonstrated that the microdialysis sampling coupled with LC–MS^n is a potentially powerful technique for achieving simultaneously rapid screening and analysis of multiple bioactive compounds in a TCM (Fructus Corni here). The methodology proposed in this paper could be applied for predicting the bioactivities of multiple components, as well as the synergic pharmaceutical activities of multiple components in the TCM, which can then be used for various pathological models. More powerful hyphenated instruments, such as HPLC–MS^n, HPLC-NMR, and high resolution-MS (HR-MS) may further aid the structural identification of absorption and metabolite components of TCMs [19,20].

Acknowledgments

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