Asian Journal of Chemistry; Vol. 23, No. 4 (2011), 1643-1647

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Liquid Chromatography Tandem Mass Spectrometry Detection of *Ablmosechus manihot* and Its Major Metabolites in Rat Urine After Oral Administration

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(*Received*: 23 June 2010; *Accepted*: 9 December 2010) AJC-9378

A highly specific and sensitive method using high performance liquid chromatography-electrospray ionization (ESI) tandem ion trap mass spectrometry(HPLC-ESI-MS^n) was developed to study on *A. manihot* and its metabolites in rat urine. The gradient elution reversed-phase HPLC system using water of 0.5 % formic acid and acetonitrile as gradient solvent mixtures was able to separate all metabolites of *Ablmosechus manihot*. The *A. manihot* were negative identified by the protonated molecules $[M-H]^-$ and further characterized by tandem mass spectrometric analysis with each it giving a characteristic collision induced dissociation product ion spectrum. Furthermore, 2 prototype constituents and 7 metabolites in urine were detected and elucidated. This convenient HPLC-MS^n method could be used to identify the chemical components of extract of *A. manihot* as well as their metabolites and to reveal their possible metabolic mechanism of action *in vivo*.

Key Words: *Ablmosechus manihot*, HPLC-MS^n, Metabolites, Urine.

INTRODUCTION

Ablmosechus manihot var. *manihot* [*Hibiscus manihot* Linnaeus] is an important traditional Chinese medicine which belongs to Malvaceae family. The seeds, roots, flowers can be used as ingredients of medicine[1]. Traditionally, it was employed for detumescence, detoxication, *etc.*[2]. Modern pharmacological experiments showed that the flower extract had a cretain therapeutic effect on nephritis and myocardial injury. It has been used for the treatment of chronic nephritis, diabetes and kidney diseases in clinical practice. It acted as a key ingredient in huangkui capsule[3,4]. Chemical investigation on *A. manihot* resulted in the discovery of several groups of bioactive components including flavonoids, steroids and fatty acids[5,6]. Modern pharmacological studies have demonstrated that flavonoids are the main bioactive compounds responsible for the nephritis and diabetes effects. Few data are available on the metabolism and metabolites of *A. manihot* extract *in vivo*. Therefore it is important to explicate the biotransformation of flavonoids *in vivo* so as to clarify the mechanism of pharmacological action and to promote its availability as well[7,8].

Chromatographic techniques coupled with mass spectrometry, such as GC-MS, LC-MS and LC-MS/MS, are the basic analytical tools for analysis of drugs and their metabolites. Chromatographic techniques, which takes the advantage in sensitivity and specificity and less time consuming and less labor intensive comparing GC and HPLC[9,10]. In addition, for clear identification in subsequent MS detection it is necessary to have good high-performance liquid chromatographic (HPLC) resolution of each metabolite which is difficult to higher selectivity and sensitivity than MS, can been used to simultaneously monitor both precursor and product ions and gives more abundant structural information for qualitative analysis and structural elucidation at trace levels[11,12].

This paper describes the development and application of a high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) for the study of the constituents and metabolites in rat urine after oral administered 2 g/kg doses of extract of *A. manihot* by *i.g.* 2 prototype constituents and 7 metabolites were found in rat urine.

EXPERIMENTAL

The flowers of *A. manihot* was purchased form medicinal materials market of Bozhou (Bozhou City, Anhui Province, China) and identified by Professor Jingui Shen. HPLC grade acetonitrile was purchased from Dikma Company (Dikma, USA). Water was deionized and bouble distilled. Other reagents used are of analytical grade.

HPLC-MS^n experiments were performed with an LCQ Duo quadru-pole ion trap mass spectrometer (Thermo Finnigan, Corp, San Jose, USA) with a modern Quat HPLC pump and an auto-sampler (Agilent 1100 series) and a TSP AS3000 auto-sampler using positive electrospray as the ionization process. The software Xcalibur version 1.2 (Finnigan) was applied for system operation and data collection. A high-speed desktop centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge urine samples. The urine samples were extracted on a C_{18} solid-phase extraction (SPE) cartridge (1 mL/100 mg, Supelco).

Chromatographic and mass spectrometric conditions: The Agilent 1100 HPLC was equipped with a reversed-phase column (Apollo-C_{18}, 4.6 mm × 250 mm i.d., 5 μm, grace), including an EasyGuard Kit C_{18} (4 mm × 2 mm) guard column. The column was maintained at 25 ºC. Detection wavelengths were set at 254 nm. The flow rate was 0.8 mL/min. A gradient elution of 0.5 % aqueous formic acid (A) and acetonitrile (B) was used as 10-20 % B at 0-5 min, 20 % B at 5-15 min, 20-30 % B at 15-20 min, 30 % B at 20-35 min, 30-60 % B at 35-50 min, 60-100 % B at 50-55 min. The mass spectra were recorded in both negative and positive modes, drying gas flow rate 10 L/min, drying gas temperature 35 ºC, nebulizer 35 psig., capillary voltage 4000 V, fragmentor 100 V, mass range 100-1500 m/z. The HPLC system was controlled by an HPLC-MSD Chemstation software system.

Animal and sample collection: Sixteen male Sprague-Dawley (SD) rats (220 ± 10 g body weigh, laboratory Animal Center of Shanghai University of Tradition Chinese Medicine) were divided into a blank group (4 rats) and drug group (12 rats). Prior to oral administration, each rat was fasted for 24 h in a metabolic cage with free access to water and were then administered 2 g/kg the extract of *A. manihot* by *i.g.* For each group, the urine of all rats was collected and admixed at 2, 4, 6, 8, 10 and 24 h. The sample were stored instantly at -80 ºC prior to analysis.

Sample preparation: Solid phase extraction (SPE) with C_{18} cartridge (1 mL/100 mg, Supelclean^{TM}, Dikma) was used to purify the above supernatants of urine sample, for LC-MS^n analysis. Before use, SPE columns were conditioned by 4 mL methanol, 2 mL deionized water. Then the selected supernatant sample was loaded and the column was washed with 3 mL of

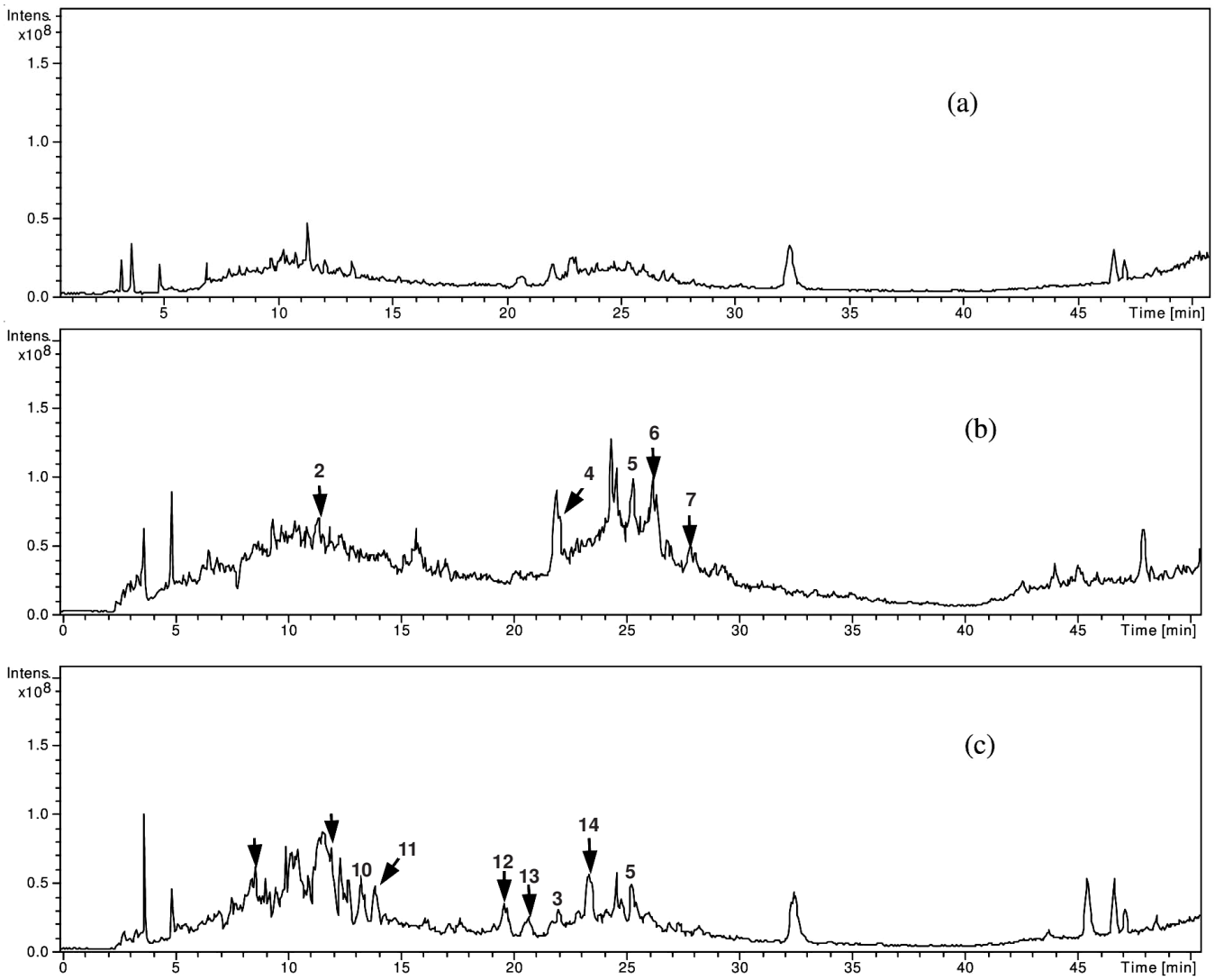

Fig. 1. HPLC-MS total ion current of the *A. manihot* decoction and its metabolites in negative mode. (a) Blank rat urine; (b) The decoction of *A. manihot*; (c) Urine sample after oral administration

deionized water to elute the impurity and 1 mL of methanol to elute the analytes in turn. The eluent was evaporated to dryness at 37 ºC in vacuum and the residue dissolved in 100 µL of 100 % methanol. After centrifugation at 12000 rpm for 10 min, 10 µL of the supernatant was introduced into the HPLC system for HPLC-MS^n.

RESULTS AND DISCUSSION

HPLC-ESI-MS^n analysis of metabolites: To elucidate the active constituents responsible for the pharmacological action, it is necessary to perceive the metabolic changes *in vivo* and chemical constituent profile in biological system. Therefore rat urine after oral administration of flavonoid extract of *A. manihot* were detected and compared with bland samples by HPLC-MS^n in negative mode. The results show that the total peaks and corresponding peak areas in metabolic chromatograms were different when collected at different periods and the most abundant metabolites were found at 8-10 h in urine samples, corresponding to the cases of components during physiological disposition. To obtain further metabolic information, the biological sample with predominant metabolic compounds were further analyzed and elucidated by HPLC-MS^n. The retetion time of each peak on HPLC, proposed compound names, $[M-H]^-$, MSD Trap fragment ions (m/z) and location of the metabolites are listed in Table-1. A total of 7 compounds were identified in the extract of *A. manihot* and 9 compounds were characterized in urine sample, including 2 original components and 7 secondary metabolites.

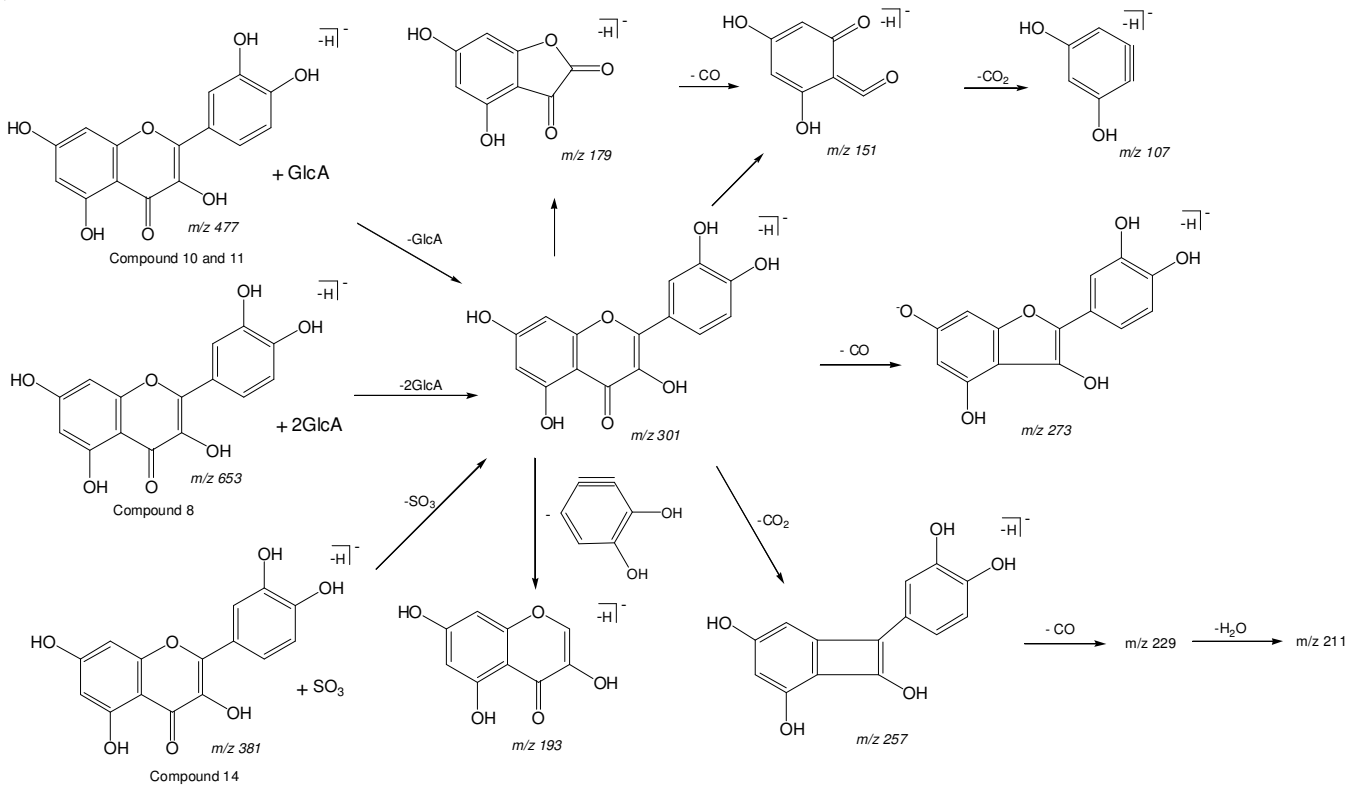

Fig. 2. Fragmentation pathways of quercetin of monoglucuronide (compounds **10** and **11**), quercetin of diglucuronide (compound 8) and quercetin sulfate conjugate (compound 14) proposed on the basis of negative ion ESI-MS^n spectra

TABLE-1
THE COMPONENTS OF *A. manihot* AND METABOLIC PROFILE OF *A. manihot* IN RAT URINE

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MS^2 fragment ions	Location
1	6.5	Gallic acid	169	125(100), 79(8), 81(6)	Plant
2	11.4	Caffeic acid	179	135(100), 107(5), 117(3)	Plant
3	21.9	Hyperoside	463	300(100), 271(25), 255(18), 179(28), 151(30)	Plant and urine
4	22.1	Isoquercitrin	463	300(100), 271(15), 255(20), 179(33), 151(25)	Plant
5	25.3	Myricetin	317	179(100), 151(34), 193(14), 109(33), 107(23)	Plant and urine
6	26.1	8-Methoxy-quercetin	331	316(100), 272(35), 256(20), 181(20), 166(20)	Plant
7	29.2	quercetin	301	179(100), 151(83), 121(41), 107(22)	Plant
8	9.5	Quercetin diglucuronide	653	477 ($[M-H]^-$-GlcA), 301($[M-H]^-$-2GlcA)	Urine
9	11.6	Myricetin monoglucuronide	493	317 ($[M-H]^-$-GlcA)	Urine
10	13.2	Quercetin monoglucuronide	477	301 ($[M-H]^-$-GlcA)	Urine
11	13.9	Quercetin monoglucuronide	477	301 ($[M-H]^-$-GlcA)	Urine
12	19.6	8-Methoxy-quercetin monoglucuronide	507	331 ($[M-H]^-$-GlcA)	Urine
13	20.6	Myricetin sulfate conjugate	397	317 ($[M-H]^-$-SO_3)	Urine
14	23.3	Quercetin sulfate conjugate	381	301 ($[M-H]^-$-SO_3)	Urine

Metabolic study in urine sample

Quercetin of glucuronide and sulfate: Compound **8** gave a $[M-H]^-$ signal at m/z 653. And m/z 653 gave the ion at m/z 477 (-176), then the successive MS/MS yielded the ion at m/z 301, involving the loss of two glucuronic acid. Compound **10** and **11** gave a $[M-H]^-$ at m/z 477, the ion at m/z 301 (-176), involving the loss of a glucuronic acid. With a retention time at 23.3 min, peak 14 generated a $[M-H]^-$ at m/z 381 in MS spectrum and a $[M-H]^-$ at m/z 301($[M-H]^-$ - 80, loss of SO_3). The MS^3 spectrum $[M-H]^-$ of the above four compounds are all 301,which is in accord with the fragmentation pathway reported in the chemical document of compound 7[13,14]. Thus,

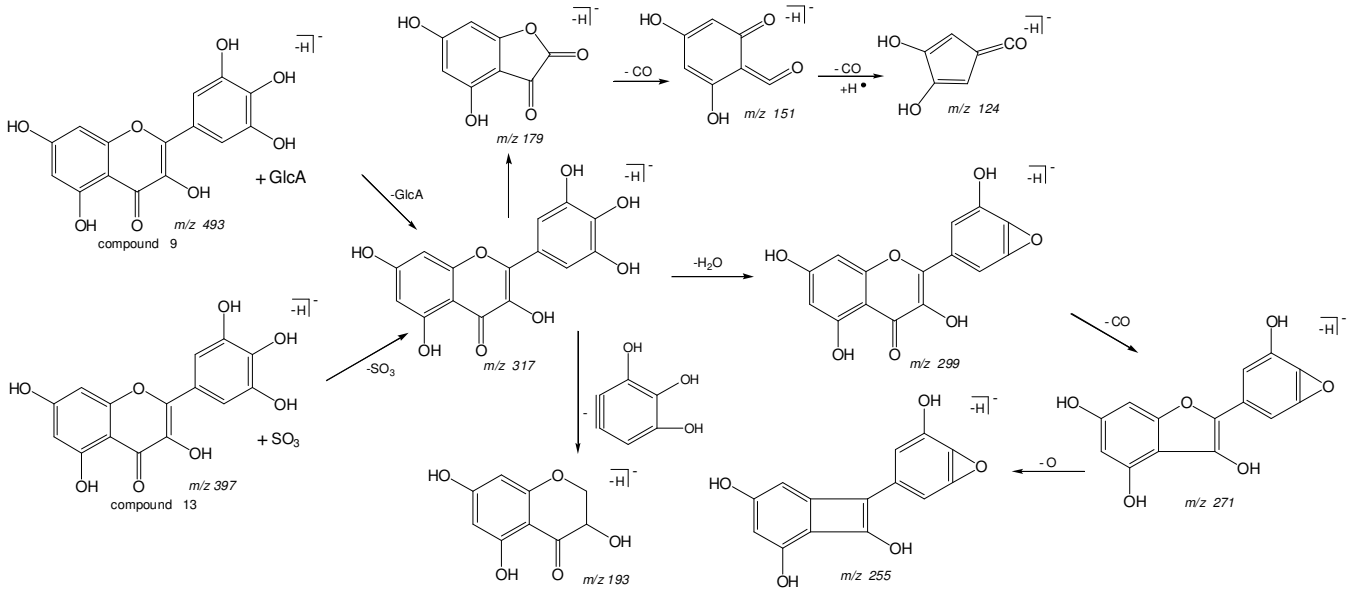

Fig. 3. Fragmentation pathways of myricetin of monoglucuronide (compound 9) and myricetin sulfate conjugate (compound 13) proposed on the basis of negative ion ESI-MS^n spectra

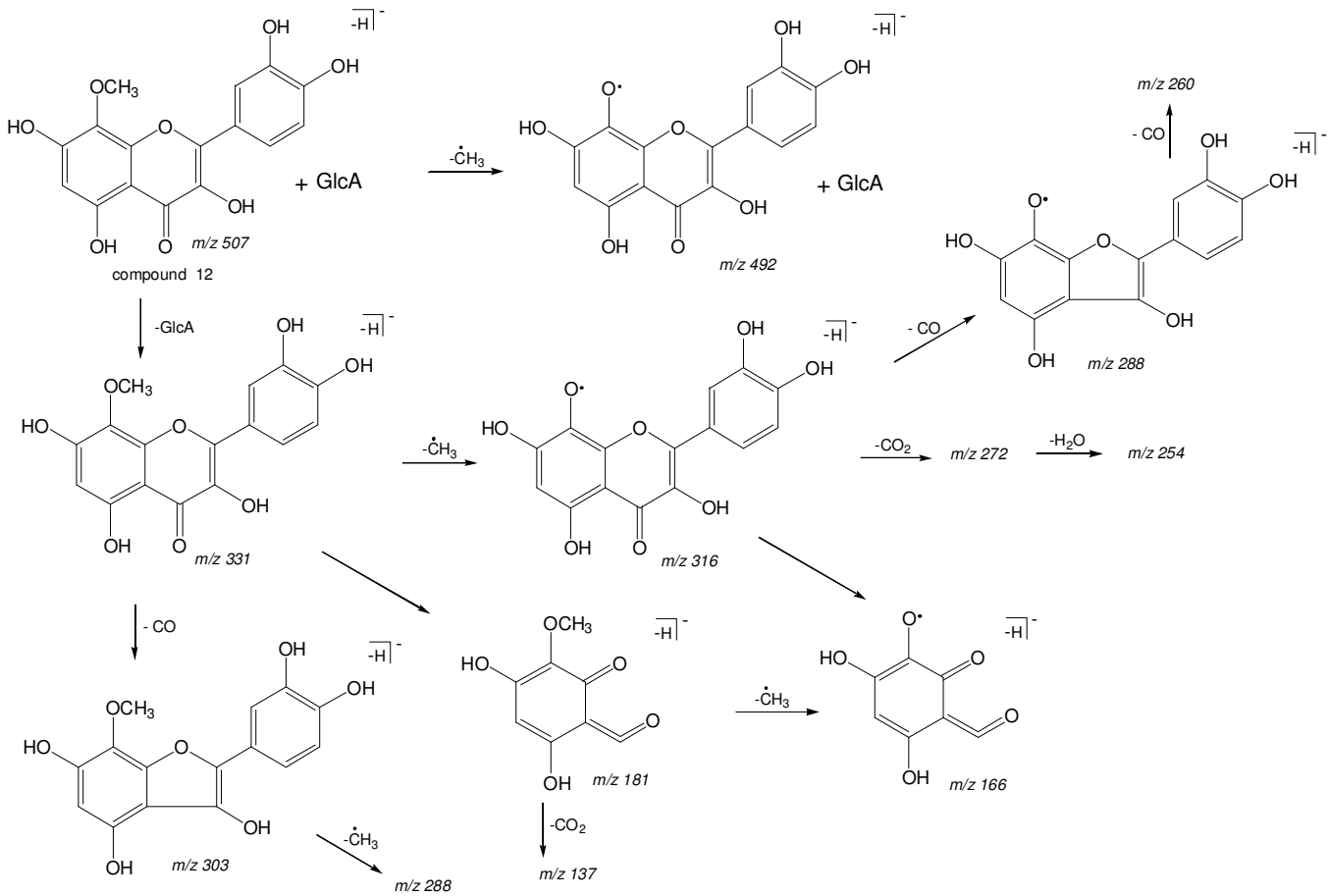

Fig. 4. Fragmentation pathways of 8-methoxy-quercetin monoglucuronide (compound 12) proposed on the basis of negative ion ESI-MS^n spectra

compounds **8**, **10**, **11** and **14** are all the metabolites of quercetin, the fragmentation pathways is presented in Fig. 2

Myricetin of glucuronide and sulfate: Compound **9** gave a $[M-H]^-$ signal at m/z 493. And m/z 493 gave the ion at m/z 317 (-176), involving the loss of a glucuronic acid. Peak 13 generated a $[M-H]^-$ at m/z 397 in MS spectrum and a $[M-H]^-$ at m/z 317($[M-H]^-$ -80, loss of SO_3). The MS^3 spectrum $[M-H]^-$ of the above two compounds are all 317 (that is to say, myricetin), which is in accord with the fragmentation pathway reported in the chemical document of compound 5[15,16], hence, compounds **9** and **14** are all the metabolites of myricetin. The fragmentation pathways is presented in Fig. 3.

8-Methoxy-quercetin of glucuronide: Peak 12, appearing at 19.6 min on HPLC, had a $[M-H]^-$ at m/z 507 and yielded a major ion at m/z 331 ($[M-H]^-$ -176Da, loss of a glucuronic acid unit) in MS^2 and MS^3 fragment $[M-H]^-$ at m/z 316 ($[M-H]^-$ -176Da-15Da, loss of a glucuronic acid unit and one neutral molecule of $CH_3\cdot$). The fragmentation pathways is presented in Fig. 4. Therefore peak 12 was presumed as 8-methoxy-quercetin glucuronide[17].

Conclusion

In this study, a simple, reliable and sensitive method has been established for the screening of main flavonoids in *A. manihot* by HPLC-MS^n. Using negative ion mode and applying the MS fragmentation rules of flavonoids reported before, 2 organic acids, 12 flavonoids, involving 5 flavonoid glycosides and 2 flavonoid sulfate conjugate, were identified or tentatively identified in ethanol extracts of *A. manihot*. However, several new analogues were identified in the present study, which proved that HPLC-MS is a powerful and rapid method to discover new constituents in Chinese medicinal herbs and its metabolites, help to lay the foundation of further study *in vivo*.

ACKNOWLEDGEMENTS

The authors thank the National Science & Technology Major Project 'Key New Drug Creation and Manufacturing Program', China (No. 2009ZX09301-001 and 2009ZX09308-005) for financial support of this work.

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