Screening and analysis of the multiple absorbed bioactive components and metabolites of Baihe Zhimu Tang by the metabolic fingerprinting technique and liquid chromatography/diode array detection-electrospray ionization-mass spectrometry

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ABSTRACT

Background: Baihe Zhimu Tang (BZT) is a widely used traditional Chinese medicinal formula in treating various diseases; however, its active components have remained unknown. Materials and Methods: Based on the metabolic fingerprinting technique and liquid chromatography/diode array detection-electrospray ionization-mass spectrometry (LC/DAD-ESI-MS), a method for rapid screening and analysis of the multiple absorbed bioactive components and metabolites of an oral solution of Baihe Zhimu Tang (BZT) in rabbit plasma, urine and feces after oral administration of BZT was developed. Results: The results obtained from a comprehensive comparative analysis of the fingerprints of the BZT and its metabolic fingerprints in rabbit biological samples indicated that 19 components in the BZT were absorbed into the rabbit’s body. Both of them were tentatively identified from their MS and UV spectra and retention behaviors by comparing the results with the reported literature. In addition, only six components were found in the metabolic fingerprints, which suggested that they might be metabolites of some components in the BZT. Conclusion: The findings demonstrated that the proposed method could be used to rapidly and simultaneously analyze and screen the multiple absorbed bioactive constituents and metabolites in a formula of traditional Chinese medicines (TCMs) by comparing and contrasting the chromatographic fingerprints with its metabolic fingerprints. This is very important not only for the pharmaceutical discovery process and the quality control of crude drugs, but also for explaining the curative mechanism of TCMs.

Key words: Absorbed bioactive components, baihe zhimu tang, liquid chromatography/diode array detection-electrospray ionization-mass spectrometry, metabolic fingerprinting

INTRODUCTION

Baihe Zhimu Tang (BZT) is a classical formula of traditional Chinese medicines (TCMs), recorded in Jinkuiyaolue written by Zhang Zhongjing around 200BC in the Han Dynasty.[1] BZT consists of Baihe (Bulbus Lilii)-Zhimu (Rhizoma Anemarrhenae) (m/m, 3:1). It was widely used for treatment of “Baihe Bing” (depression) and menopausal syndrome in clinical practice in China. Our previous research also has revealed that BZT has the effects of regulating the level of central neurotransmitters including dopamine, 5-hydroxytryptamine and norepinephrine in rat brain and significantly relieving menopausal symptoms in rat model.[2,3] The phenolic glycosides (regaloside A, regaloside B), saponins (timosaponin B, timosaponin AIII, timosaponin E, gracillin), and xanthone glycosides (neomangiferin, mangiferin) are the main components in BZT.[4] In particular, mangiferin and regaloside A were used...
in fingerprint quality control of BZT.\[5\] The structures of some components of BZT are shown in Figure 1.

TCMs is a very complex mixture containing hundreds or even thousands of components, which makes it extremely difficult to screen and analyze the bioactive components contained in it. In traditional approaches, a single component isolated from TCMs is tested one by one on animal models to screen bioactive components.\[6\] The constituents in TCMs are so complicated that it is not easy to separate all the single components. Thus, the traditional methods seem to be time-consuming, arduous, and unsuitable for TCMs. In recent years, the development of modern biological techniques has provided new techniques and approaches for the modern studies on bioactive components in TCMs. Whole animal models (e.g., serum pharmacology),\[7\] receptor models and molecular biochromatography,\[8\] cell models, and cell membrane chromatography\[9-11\] have all been used to screen the bioactive components in TCMs. However, these methods can only simulate the biological environment to predict what would happen in vivo and cannot reflect the real circumstances of complex organisms.

Figure 1: Chemical structures of components identified in an oral solution of Baihe Zhimu Tang
In order to project the integrity and synergistic effects of TCMs, chromatographic fingerprinting[12-14] was proposed as a technique to examine the composition and efficacy, by the use of hyphenated instrumentation such as gas chromatography/mass spectrometry (GC/MS), high performance liquid chromatography/diode-array detection (HPLC/DAD), capillary electrophoresis (CE)-MS, and LC/DAD-MS.[15-18] Metabolic fingerprinting is the combination of metabolite profiling and the chromatographic fingerprinting technique, which not only reflects what can happen in vivo, but also projects the integrity of TCMs. So, the metabolic fingerprinting technique is suitable for screening and analysis of the bioactive components of TCMs. Today, the use of the LC-MS technique in metabolic fingerprinting is advancing and there are a few publications in the field of TCMs.[19-20] However, to our best knowledge, application of the metabolic fingerprinting and LC/DAD-ESI-MS approach to screen and analyze the multiple absorbed bioactive components and metabolites of BZT in rabbit has not been reported.

In the present study, a new method based on the combination of metabolic fingerprinting and the LC/DAD-ESI-MS technique is proposed to rapidly screen and analyze the multiple absorbed bioactive components and metabolites of BZT in vivo. Absorbed bioactive components and metabolites were analyzed by comparing and contrasting the components measured in the chromatographic fingerprints of the BZT with those in its corresponding metabolic fingerprints. Based on the techniques, 19 bioactive components were tentatively identified from their MS, UV spectra, and retention behaviors by comparing the results with those reported in the literature.[4] The proposed technique made it possible to rapidly screen and analyze the multiple bioactive components and metabolites in a formula of TCMs.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Timosaponin AIII was isolated from Zhimu in the authors’ laboratory. The structure was elucidated by their spectral data (MS, 1H NMR, and 13C NMR).[23] The purity was determined to be higher than 98% by HPLC. Baihe was purchased from Yixing City, Jiangsu Province and Zhimu was purchased from Yixian, Hebei Province. All samples were identified by Professor Jianwei Chen from Nanjing University of Chinese Medicine. The voucher specimens were deposited in our laboratory. Acetonitrile (Tedla, USA) and formic acid (Tedla, USA) were HPLC grade. Other reagents were of analytical grade and all water used was Milli-Q grade (Millipore, Bedford, MA, USA).

**Instrumentation**

The HPLC system consisted of a Shimadzu LC-20AD pump, an SIL-20A auto sampler, a CTO-20A column oven, a DGU-20A degasser, and an SPD-M20A diode-array detector (Shimadzu, Kyoto, Japan). The mass spectrometer was an LC-MS-2020 single quadrupole equipped with an electrospray ionization (ESI) source interface (Shimadzu). A Speed Vacplus model vacuum drier (Buchi, Sweden) was used in the preparation of samples. The data processing was carried out using LCMS Solution software (Shimadzu, Kyoto, Japan).

**Liquid chromatography, diode array detection, and mass spectrometry conditions**

For chromatographic analysis, an Shim-Pack VP-ODS C18 column (150 mm × 2.0 mm, 5 μm) with a suitable guard column (ODS C18, 5 μm, 4 × 3.0 mm) was used. HPLC separation was performed using a linear gradient at room temperature (35°C) and a flow rate of 0.2 mL/min. The mobile phase consisted of acetonitrile (A) and water containing 0.05% formic acid (B) using the elution gradient 8-15% A at 0-30 minutes, 15-22% A at 30-40 minutes, 22-28% A at 40-60 minutes, 28-35% A at 60-75 minutes, 35-55% A at 75-90 minutes, 55-80% A at 90-95 minutes, and 80-8% A at 95-100 minutes. Detection wavelength was set at 315 nm.

The ESI-MS spectra were acquired in both negative and positive modes scanning from 100 to 1300. The typical ion source parameters were as follows: ESI probe temperature 350°C, CDL temperature 280°C, heat block temperature 320°C, ESI probe voltage 4.5 kV, detector voltage 1.5 kV, DL voltage 50 V, Q-array DC voltage 50 V, Q-array RF voltage 120 V, and nebulizing gas flow 1.5 L/min. Tuning of the mass spectrometer was accomplished with the help of the autotuning function of LCMS Solution software using the tuning standard solution (polypropylene glycol). Optimization and calibration of the mass spectrometer were achieved with autotuning.

**Biological samples collection**

The samples of TCMs were crushed to a homogeneous size in a mill, sieved through a no. 40 mesh. Baihe (300 g) and Zhimu (100 g) were immersed in 4000 mL (10 times their total weight) deionized water overnight and then boiled for 1 hour for the first decoction. The extract was filtered through a four-layer bandage while it was hot. This procedure was then repeated using another 4000 mL deionized water. The two extracts were combined and concentrated by rotary evaporator to approximate 500 mL. Half milliliter of the extract was then added with 2 mL ethanol to precipitate the polysaccharide and protein. Being stirred and allowed to stand for 24 hours, the mixture was filtered using analytical filter paper. The
filtrate was collected and concentrated to dryness by rotary vaporization at 60°C under reduced pressure. At last, the dried residue was dissolved with distilled water into a 200 mL volumetric flask as BZT stock solution (2.0 g crude drug/mL).

A healthy rabbit (2.3 kg) was provided by Laboratory Animal Center of Nanjing University of Chinese Medicine. Prior to oral administration of the above BZT stock solution, the animal was fasted in metabolic cage for 3 days maintaining with physiological saline and fed with standard laboratory food and water ad libitum. During this period, the blank plasma, urine, and feces samples were collected and stored at −20°C prior to analysis. Then, BZT was administered orally to the rabbit at a dose of 20 g/kg twice a day for 3 days. The urine and the feces of rabbit within 72 hours were collected and stored at −20°C until analysis. Before the last administration, the rabbit was deprived of food for 12 hours once more, and then 1 hour later the blood was collected from the rabbit ear vein and pipetted into a glass tube. The blood was then centrifuged at 4000 rpm for 10 minutes to separate plasma, and the obtained plasma sample was stored at −20°C prior to analysis. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, revised edition 2004).

Processing and analysis of biological samples
The plasma sample (5 mL) was placed into a 50 mL polypropylene tube, and 15 mL of methanol was added to the tube. The mixture was vortexed for 120 seconds. The precipitated protein was removed by centrifugation at 13,000 rpm for 10 minutes. The organic layer was transferred to another tube and evaporated to dryness at 40°C in the Speed Vacplus vacuum drier and stored at −20°C until analysis. The urine sample (20 mL) was treated with 95% (v/v) ethanol in the proportion of 1:3. It was then stirred and allowed to stand for 1 hour at −4°C. The mixture was filtered and the filtrate was evaporated to dryness at 40°C in the Speed Vacplus vacuum drier, and the residue was dissolved in 5 mL 50% (v/v) methanol. The eluted solution was filtered through a 0.45 μm membrane filter. A 5 μL aliquot of the filtrate was injected into the HPLC system for LC-MS analysis.

The feces sample (5 g), after stirred with a glass rod into powder, was extracted with 50 mL 75% (v/v) methanol in an ultrasonic bath for 60 minutes successively, followed by filtration. The filtrate was evaporated to dryness at 40°C in the Speed Vacplus vacuum drier, and the residue was dissolved in 5 mL 50% (v/v) methanol. The eluted solution was filtered through a 0.45 μm membrane filter. A 5 μL aliquot of the filtrate was injected into the HPLC system for LC-MS analysis.

RESULTS AND DISCUSSION

Selection of mass conditions
In our preliminary research, we have set up the HPLC and mass conditions for analysis of BZT,[4] so we use similar conditions in this research with a little change on the scan range. During selection of the scan range, timosaponin AIII as one of the bioactive components in Zhimu (molecular weight (MW) 740) was taken into account. Firstly, the scan range m/z 100-1500 was selected to detect timosaponin AIII as a reference compound. The result showed that the m/z of the base peak in the positive mode was 763 in this condition. To reduce noise, the scan range should be decreased. To our best knowledge, the maximum m/z of small molecules in the reported papers about Baihe and Zhimu was 1064. So, the scan range m/z 100-1300 was selected to measure multiple components in BZT.

Our former trials also showed that saponins can give better chromatograms in positive mode than in negative mode, while xanthone glycosides and phenolic glycosides showed better chromatograms in negative mode.[4] To get more information of all compounds in MS spectra, the analysis was carried out in both positive and negative mode.

Analysis of the chromatographic fingerprint of BZT and different metabolic fingerprints after oral administration of BZT
The rapid screening and analysis of bioactive components in TCMs is very important not only for the pharmaceutical discovery process and the quality control of crude drugs, but also for explaining the curative mechanism of TCMs. However, it was neither possible nor necessary to isolate every single component in a formula of TCMs. Instead, a “fingerprint” of a formula of TCMs can be a more feasible route. In the same way, during analysis of the bioactive components of a formula of TCMs, a “metabolic fingerprint” of a formula of TCMs might be a more feasible technique. By comparing chromatographic fingerprint and different metabolic fingerprints of a formula of TCMs, one might simultaneously and rapidly screen and analyze the multiple absorbed bioactive components and metabolites of a formula of TCMs.

Before studying the multiple absorbed bioactive components and metabolites of BZT in rabbit after oral administration,
the original main components in BZT were identified by LC/DAD-ESI-MS in both negative and positive modes. By attentive study of the mass spectra of these compounds and comparison with standards and reference data, a total of 38 major components including 31 saponins, 4 phenolic glycosides and 3 xanthone glycosides were designated and identified.[4]

The LC/DAD-ESI-MS chromatographic fingerprints of plasma, urine, and feces samples before and after oral administration of BZT, namely metabolic fingerprints. Most components in BZT do not possess any or only weak UV chromophores. So, DAD was not suitable to detect those components. However, mass spectra could resolve this problem. More peaks and higher responses can be detected in the LC-ESI-MS metabolic fingerprints postdose than metabolic fingerprints of blank samples. So, LC-ESI-MS metabolic fingerprints were used to compare and contrast with the chromatographic fingerprint of BZT to study the multiple absorbed bioactive components and metabolites of BZT in rabbit.

In order to get more information, we carefully compare the chromatographic fingerprint of BZT, metabolic fingerprints of blank biological samples and metabolic fingerprints after oral administration of BZT. It was found that 19 peaks were common both in BZT chromatographic fingerprint and the metabolic fingerprints of different biological samples by comparing their MS spectra, UV spectra and corresponding retention times. The MS spectra, UV spectra, and retention times of 19 peaks for common components are listed in Table 1. This information will be very important for the pharmaceutical discovery process and quality control of crude TCMs. The other six peaks were only detected in the metabolic fingerprints [Table 2], which indicated that those components were metabolites of some components in BZT. This information may be of great potential significance for better understanding of pharmacologic actions of BZT from the chemical viewpoint.

Analysis of the multiple absorbed bioactive components and metabolites in rabbit biological samples

By comparing the MS chromatograms of rabbit biological samples after oral administration of BZT with blank rabbit biological samples in both positive mode and negative mode, 25 peaks were observed in dosed rabbit biological samples but not in blank rabbit biological samples. Among the 25 peaks, 19 peaks were also found in the MS spectra of BZT in both positive mode and negative mode, which indicated that these components were absorbed into rabbit body in the prototype. By comparing the retention times and MS data with the literature data[16] or reference standards, these 19 compounds, including 16 saponins, 2 xanthone glycosides, and 1 phenolic glycoside were all identified.

In rabbit plasma, 12 absorbed components and 4 metabolites were detected, and the LC-ESI-MS total ion current chromatograms in negative ion mode for rabbit plasma are shown in Figure 2. Nine original absorbed components were identified as saponins, two original absorbed components were identified as xanthone glycosides, and one original absorbed components were identified as phenolic glycosides by comparing their UV spectra, their molecular weights and their retention times with those data of BZT.[4,26,27] Among the four metabolites detected in rat plasma, only M21 may be degraded from xanthone glycosides in Zhimu. By comparing the MS spectrum and the UV spectrum, M21 (MW436) was presumed to be the monomethyl conjugate of mangiferin, which might have originated from the metabolite of mangiferin or related skeleton structures in vivo.

In rabbit urine, 13 original absorbed components and 5 metabolites were detected, and the LC-ESI-MS total ion current chromatograms in positive ion mode for rat urine are shown in Figure 3. Among these original components, regaloside A is phenolic glycoside, the other 12 components were identified as saponins.[4,26,27] Among the five metabolites detected in rat urine, M20 (MW612) was tentatively identified as the monomethyl conjugate of mangiferin, which might have originated from the metabolite of mangiferin or related skeleton structures in vivo.[26] M21 (MW436) was presumed to be the monomethyl conjugate of mangiferin.[26] No original components of xanthone glycosides were detected in rat urine, the reason can possibly be that the xanthone glycosides may degrade and transform quickly into other compounds. Further investigation of the reason is now being carried out in our laboratory.

In rabbit feces, 10 original absorbed components and one metabolite were detected, and the LC-ESI-MS total ion current chromatograms in negative ion mode for rat feces are shown in Figure 4. Among these original absorbed components, nine known saponins and one xanthone glycoside were tentatively identified according to characteristics of molecular weight and LC-ESI-MS data of BZT.[4,26,27] The metabolite detected at 87.347 minutes gave the base peaks at m/z 783 [M-H]− in negative mode and 785 [M + H]⁺ in positive mode. Therefore, m/z 784 was considered as its molecular weight. The identification of this metabolite is still in progress.

Identifying the detected compounds

Due to the fact that it offers more information, including the MWs and the UV spectra as well as the retention behaviors...
### Table 1: Liquid chromatography-electrospray ionization-mass spectrometry data for original absorbed components of Baihe Zhimu Tang in rabbit biological samples

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time $t_r$ (minutes)</th>
<th>In negative mode</th>
<th>In positive mode</th>
<th>Molecular weight</th>
<th>Compound presumed</th>
<th>Plasma</th>
<th>Urine</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[M-H]</td>
<td>[M+HCOO]$^-$</td>
<td>[M+H]$^+$</td>
<td>[M+Na]$^+$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>12.150</td>
<td>583</td>
<td>607</td>
<td>584</td>
<td>Neomangiferin</td>
<td>+</td>
<td></td>
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<tr>
<td>M2</td>
<td>20.102</td>
<td>421</td>
<td>445</td>
<td>422</td>
<td>Mangiferin</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>M3</td>
<td>22.132</td>
<td>399</td>
<td>423</td>
<td>400</td>
<td>Regaloside A</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>45.254</td>
<td>935</td>
<td>959</td>
<td>936</td>
<td>Timosaponin E1 or N or their isomers</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>M5</td>
<td>46.817</td>
<td>935</td>
<td>959</td>
<td>936</td>
<td>Timosaponin E1 or N or their isomers</td>
<td>+</td>
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<td>M6</td>
<td>48.259</td>
<td>917</td>
<td>941</td>
<td>918</td>
<td>Timosaponin D or its isomers</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>M7</td>
<td>50.150</td>
<td>917</td>
<td>941</td>
<td>918</td>
<td>Timosaponin D or its isomers</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>M8</td>
<td>53.463</td>
<td>933</td>
<td>934</td>
<td>920</td>
<td>Timosaponin Ba or its isomers</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>M9</td>
<td>54.023</td>
<td>919</td>
<td>920</td>
<td>919</td>
<td>Timosaponin Ba or its isomers</td>
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<td>+</td>
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<tr>
<td>M10</td>
<td>55.473</td>
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<td>943</td>
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<td>Timosaponin Ba or its isomers</td>
<td>+</td>
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<tr>
<td>M11</td>
<td>59.605</td>
<td>901</td>
<td>925</td>
<td>902</td>
<td>Timosaponin B or its isomers</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>M12</td>
<td>60.067</td>
<td>901</td>
<td>925</td>
<td>902</td>
<td>Timosaponin B or its isomers</td>
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<tr>
<td>M13</td>
<td>72.133</td>
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<td>925</td>
<td>902</td>
<td>Timosaponin B or its isomers</td>
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<tr>
<td>M14</td>
<td>73.390</td>
<td>901</td>
<td>925</td>
<td>902</td>
<td>Timosaponin C</td>
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<td>+</td>
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<tr>
<td>M15</td>
<td>78.214</td>
<td>883</td>
<td>884</td>
<td>884</td>
<td>Gracillin</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>M16</td>
<td>82.447</td>
<td>757</td>
<td>781</td>
<td>758</td>
<td>Timosaponin I</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>M17</td>
<td>86.253</td>
<td>755</td>
<td>779</td>
<td>756</td>
<td>Timosaponin All or G</td>
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<td></td>
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<tr>
<td>M18</td>
<td>91.359</td>
<td>739</td>
<td>763</td>
<td>740</td>
<td>Timosaponin $B\alpha$</td>
<td>+</td>
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<tr>
<td>M19</td>
<td>93.597</td>
<td>739</td>
<td>763</td>
<td>740</td>
<td>Timosaponin $B\alpha$</td>
<td>+</td>
<td></td>
<td></td>
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</tbody>
</table>

+Detected

### Table 2: Liquid chromatography-electrospray ionization-mass spectrometry data for metabolites of Baihe Zhimu Tang in rabbit biological samples

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time $t_r$ (minutes)</th>
<th>In negative mode</th>
<th>In positive mode</th>
<th>Molecular weight</th>
<th>Plasma</th>
<th>Urine</th>
<th>Feces</th>
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<tr>
<td></td>
<td></td>
<td>[M-H]</td>
<td>[M+H]$^+$</td>
<td>[M+Na]$^+$</td>
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<td></td>
<td></td>
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<tr>
<td>M20</td>
<td>14.837</td>
<td>611</td>
<td>635</td>
<td>612</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>M21</td>
<td>24.533</td>
<td>435</td>
<td>437</td>
<td>436</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>M22</td>
<td>42.837</td>
<td>623</td>
<td>647</td>
<td>624</td>
<td>+</td>
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<tr>
<td>M23</td>
<td>65.953</td>
<td>639</td>
<td>641</td>
<td>640</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>M24</td>
<td>87.347</td>
<td>783</td>
<td>785</td>
<td>784</td>
<td>+</td>
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<td></td>
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<tr>
<td>M25</td>
<td>92.847</td>
<td>893</td>
<td>895</td>
<td>894</td>
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</tbody>
</table>

+Detected
of the detected compounds, LC/DAD-ESI-MS was thought to a powerful tool to identify chemical structures. Thus, the peaks can be identified by comparing their MS spectra, their MWs, and their retention behaviors with data reported in the literature.[4] At least 19 components seem to be tentatively identified: neomangiferin (M1), mangiferin (M2), regaloside A (M3), timosaponin E1 or N or their isomers (M4, M5), timosaponin D or its isomers (M6, M7), timosaponin E or BI (M8), timosaponin Bα or its isomers (M9, M10), 26-O-β-D-glucopyranosyl-3β,26-dihydroxycholestan-16,22-dioxo-3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside or its isomer (M11, M12), timosaponin B (M13), timosaponin C (M14), gracillin (M15), timosaponin I (M16), timosaponin AII or G (M17), timosaponin BIV (M18), and timosaponin A III (M19).

M9 was used as an example to explain the procedure of identification. Firstly, the mass spectrum was examined. M9 was determined to be the saponin compound from its mass spectrum showing the presence of [M+Na]\(^+\) in positive mode and [M+HCOO]\(^-\) in negative mode. As can be seen from [Figure 5], m/z 943 [M+Na]\(^+\), and 903 [M-H\(_2\)O+H]\(^+\) in positive mode matched with that of timosaponin Bz or its isomers reported in the literature.[4] In the negative mode, M9 gave an [M-H]\(^-\) ion at m/z 919, and an [M+HCOO]
bioactive components were tentatively identified through analyzing their MS, UV spectra, and retention behaviors and also comparing the results with the reported literature. The method made it possible to rapidly and simultaneously screen and analyze the multiple absorbed bioactive components and metabolites in a formula of TCMs.

REFERENCES


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Announcement

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