

RESEARCH ARTICLE

Screening and Identification of the Metabolites of Water Extracts of Raw and Honey-Processed *Astragalus* in Rat Urine Based on UHPLC/ESI-Q-TOF-MS and Multivariate Statistical Analysis

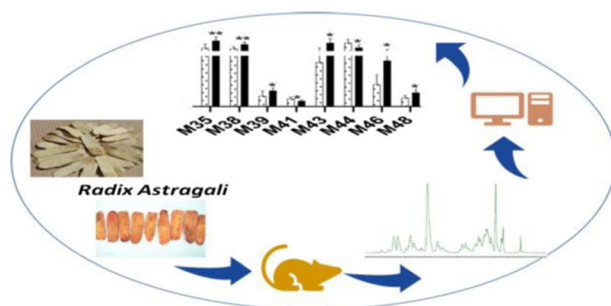
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Abstract. *Radix Astragali* is a famous traditional Chinese medicine and honey-processed *Astragalus* is a product of *Radix Astragali* acquired by honey-processing. These two products are widely utilized to treat various diseases. In this study, we screened bioactive components and metabolites of raw and honey-processed *Astragalus* in rat urine by ultra-performance liquid chromatography equipped with electrospray ionization/quadrupole time-of-flight mass spectrometry (UHPLC/ESI-Q-TOF-MS) combined with multivariate statistical analysis.

In total, 62 compounds, including 7 parent compounds and 55 metabolites, were detected and 11 metabolites were characterized for the first time. The identified metabolites indicated that the metabolic reactions of *Astragalus* in rats included hydroxylation, glucuronidation, deglycosidation, monomethylation, demethylation, sulfation, hydrogenation, and dehydroxylation. The metabolic pathways of raw and honey-processed *Astragalus* in rat urine also were clarified. Through multivariate statistical analysis of the data of the raw and honey-processed *Astragalus* groups, we found that 20 compounds were differential components and that 1 metabolite only existed in the honey-processed *Astragalus* group. The differences in these ingredients between these two groups might provide the basis for interpreting the biologic activity differences in traditional Chinese medicine treatments.

Keywords: *Radix Astragali*, Honey-processed, UHPLC/ESI-Q-TOF-MS, Multivariate statistical analysis, Metabolite

Abbreviations UHPLC/ESI-Q-TOF-MS, Ultra-high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry; PCA, Principal component analysis; OPLS-DA, Orthogonal partial least squares-discriminant analysis; PLS-DA, Partial least squares-discriminant analysis; EIC_s, Extracted ion chromatograms; *m/z*, Mass-to-charge ratio; BPI, Base peak ion chromatograms; MS/MS, Tandem mass spectrometry

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Introduction

Radix Astragali is a well-known traditional Chinese medicine and has been extensively used in clinical application in China. It is the dried root of *Astragalus membranaceus* (Fisch.) Bge. and *Astragalus membranaceus* (Fisch.) Bge. var. *mongolicus* (Bge.) Hsiao, and also called Huang Qi in Chinese [1]. Honey-processed *Astragalus* is a composite product of *Radix Astragali* and honey prepared by a traditional processing method. Honey-processed *Astragalus* is utilized to replace raw *Radix Astragali* to achieve in reinforcing “Qi” (vital energy) and the latter is mainly used to induce diuresis, alleviate edema, promote pus discharge, and tissue regeneration [2, 3]. Pharmacological and clinical studies documented that *Radix Astragali* had various effects, such as immunomodulating and immunorestorative effects [4], anti-tumor effects [5], antioxidative effects [6], and cardiovascular function enhancement [7]. Honey-processed *Astragalus* improves the protection of damaged red blood cells and immunologic function [3, 8], but reduce the antioxidant effects [9]. However, the differences in pharmacological effects of these two products are unknown.

Only absorbed ingredients are given the opportunity to become bioactive constituents [10]. Therefore, it is necessary to explore the serum pharmacochimistry of *Radix Astragali* in order to find bioactive ingredients and differences in vivo between raw and honey-processed *Astragalus*. Actually, the contents of bioactive ingredients absorbed into blood are so low that it is sometimes difficult to detect these ingredients. However, the contents of bioactive ingredients in urine are higher than those in serum. In the past decade, in vivo metabolism of *Radix Astragali* or other prescriptions containing *Radix Astragali* are reported [11–18], but in vivo metabolites of honey-processed *Astragalus* are seldom explored. In *Radix Astragali* oral solution and injection, which are widely used to treat numerous diseases, the main ingredients are water extracts of *Radix Astragali* [19]. Up to now, the comparative study on the metabolites of water extracts of raw and honey-processed *Astragalus* has not been reported. Therefore, it is necessary to evaluate the metabolites of water extracts of raw and honey-processed *Astragalus* and compare their absorption and metabolism differences.

Active components in *Radix Astragali* mainly include isoflavonoids, saponins, and polysaccharide [11]. The differences in pharmacological effects between the raw and processed *Astragalus* are possibly caused by the changes in the chemical composition of *Radix Astragali* after honey-processing. In our previous study, the fingerprint of honey-processed *Astragalus* was established by UHPLC/ESI-Q-TOF-MS and the differences in the chemical constituents between the raw and honey-processed *Astragalus* in vitro were characterized [20]. This paper aims to study the metabolic difference in vivo in the active constituents of these two products. UHPLC-MS/MS is a powerful technique to detect metabolites in biological fluids. However, this technique is being challenged due to ion suppression or interferences from the sample matrix, especially the sample matrix which interferes with the

identification of metabolites [21]. In order to detect more metabolites of drugs in rat urine and eliminate matrix effects, we developed a sensitive and reliable UHPLC/ESI-Q-TOF-MS method combined with multivariate statistical analysis-principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) to analyze the metabolites in urine samples. The active ingredients of raw and honey-processed *Astragalus* were compared and the metabolite differences were explored by PCA and OPLS-DA for interpreting biologic activity differences in traditional Chinese medicine treatments.

Experimental

Materials and Reagents

Astragalus membranaceus (Fish) Bungevar. *Mongholicus* (Bunge) Hsiao was purchased from Guangzhou Caizhilin Pharmaceutical Co., Ltd. (Guangdong, China) and further confirmed by Professor Jizhu Liu from Guangdong Pharmaceutical University. Calycosin, formononetin, and ononin (purity > 98%) were purchased from Chengdu Purechem-Standard Co., Ltd. (Sichuan, China). Astragaloside IV, astragaloside I and calycosin-7-O- β -D-glucoside (purity > 98%) were obtained from Chengdu Push Bio-technology Co., Ltd. (Sichuan, China). β -Glucuronidase was purchased from Sigma-Aldrich (USA). Acetonitrile and methanol of HPLC grade were from Merck (Darmstadt, Germany). HPLC-grade acetic acid was acquired from CNW Technologies GmbH (Düsseldorf, Germany). Water used for the UHPLC/ESI-Q-TOF-MS analysis was purchased from Watsons (Guangzhou, China).

Animals

Male Sprague-Dawley rats 200 ± 20 g were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All rats were acclimatized in a fixed environment for 1 week and fasted with free access to water for 12 h before the experiment. The room temperature was controlled at 25 ± 2 °C; relative humidity was $50\% \pm 10\%$; the light-dark cycle was 12:12 h. All experimental protocols had been approved by the Institutional Animal Ethics Committee of Guangdong Pharmaceutical University. Animals were randomly divided into control group, raw *Astragalus* group, and honey-processed *Astragalus* group before the experiment and each group with seven rats.

Preparation of Water Extracts of Raw and Honey-Processed *Astragalus* and Standard Solution

The standards were accurately weighed and then dissolved with methanol to prepare the stock solutions. The concentrations of calycosin, formononetin, ononin, astragaloside IV, astragaloside I, and calycosin-7-O- β -D-glucoside in the stock solution were respectively 0.170, 0.164, 0.200, 0.135, 0.200, and 0.477 mg/mL; the stock solutions were kept at 4 °C.

Radix Astragali were crushed and 400 g powders were weighed and soaked in 4 L of water for 30 min. Then water extracts were boiled for 1 h and filtrated with gauze. Next, the drugs were boiled twice for 1 h respectively with 3.2 and 2.4 L of water, then the extracts obtained in two times were respectively filtrated out according to the above methods. All the obtained filtered solution was merged and condensed to 300 mL by heating and then placed in a $-80\text{ }^{\circ}\text{C}$ refrigerator overnight. In the next day, water extracts were freeze-dried in Alpha 1-4 LD PLUS freeze-drying machines (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) for 24 h. The honey-processed *Astragalus* was acquired according to the method described in Chinese Pharmacopoeia 2015 [22]. Water extracts of honey-processed *Astragalus* was prepared according to the above methods. The raw and honey-processed *Astragalus* freeze-dried powder was kept at $-20\text{ }^{\circ}\text{C}$ for further use.

Sample Preparation

Raw and honey-processed *Astragalus* freeze-dried powders were formulated with distilled water before oral administration. Rats of raw and honey-processed *Astragali* groups were twice-daily administered by intragastric gavage for 3 consecutive days. The administration dose was equivalent to 30 g crude drug/kg and 2 mL per 100 g body weight. The equivalent volume of distilled water was administrated to the control group. Urine samples were collected in metabolism cages within 24 h after the last administration, then centrifuged for 20 min (4000 rpm, $4\text{ }^{\circ}\text{C}$) and the supernatants were stored at $-80\text{ }^{\circ}\text{C}$ for analysis.

Urine samples (each group has 7 parallel samples) were treated with methanol before LC/MS analysis. Firstly, 0.5 mL of rat urine was transferred into a centrifuge tube and then vortexed with 0.5 mL methanol for 1 min. Then, the mixture was centrifuged for 30 min (13,000 rpm, $4\text{ }^{\circ}\text{C}$). The supernatant was transferred into another tube and evaporated to dryness under the stream of nitrogen at $30\text{ }^{\circ}\text{C}$. The residue was reconstituted in 250 μL of the methanol/water solution (*V/V*, 50%:50%), fully vortexed, and then centrifuged for 30 min (13,000 rpm, $4\text{ }^{\circ}\text{C}$). The supernatant was used only for further UHPLC/ESI-Q-TOF-MS analysis.

Enzymatic Hydrolysis Sample Preparation

Firstly, 0.5 mL drug-containing urine was mixed with β -glucuronidase solution (1000 U/mL, dissolved in acetic-sodium acetate buffer which was adjust to pH 6.8 with acetic acid), and then enzymatic hydrolysis carried out at $37\text{ }^{\circ}\text{C}$ for 24 h. The sample processing method was the same as mentioned above.

Instrumentation and Experimental Conditions

ACQUITY UHPLC/Q-TOF Microsystems (Waters Co., Massachusetts, USA) was utilized in the study. Chromatographic separation was performed at $25\text{ }^{\circ}\text{C}$ on a Phenomenex Kinetex EVO C18 column ($50 \times 2.1\text{ mm}$, $1.7\text{ }\mu\text{m}$, Phenomenex, Torrance, USA). The mobile phase consisted of 0.2% acetic acid water (A) and acetonitrile (B). The gradient elution was as follows: 0–

3 min, 2–20%B; 3–7 min, 20%B; 7–8 min, 20–25%B; 8–16 min, 25–45%B; 16–17 min, 45–50%B; 17–18 min, 50–100%B; 18–19 min, 100%B; 19–20 min, 100–2%B; 20–22 min, 2%B. The injection volume was 2 μL and the flow rate was at 0.3 mL/min.

In mass analysis, the centroid mode was adopted and the mass range set at 100–1500 mass-to-charge ratios (*m/z*) in both positive and negative ionization modes. Nitrogen and argon were used as cone and collision gas, respectively. The parameters of ESI source were as follows: the flow rate of nebulizer gas (N_2), 50 L/h; the flow rate of desolvation gas (N_2), 600 L/h; desolvation temperature, $300\text{ }^{\circ}\text{C}$; source temperature, $120\text{ }^{\circ}\text{C}$; capillary voltage, 3000 V; sample cone, 30 V; extraction cone voltage, 4.0 V (positive mode) and 2.0 V (negative mode). In the tandem mass spectrometry (MS/MS) experiments, variable collision energy (15–40 eV) was optimized for individual compound. In order to ensure the mass accuracy and reproducibility, leucine enkephalin ($[\text{M}+\text{H}]^+ m/z\ 556.2771$; $[\text{M}-\text{H}]^- m/z\ 554.2615$; 1000 ng/mL) was used as a reference.

Data Processing and Analysis

As shown in Figure 1, all the data of the tested samples were analyzed in MarkerLynx™ software (Version 4.1, Waters Co., Cicero, USA) and SIMCA-P 12.0 software (Version 12.0, Umetrics, Umeå, Sweden). According to the edited MarkerLynx method, UHPLC/ESI-Q-TOF-MS data was obtained as the input data of the SIMCA-P software. Retention time range was set at 0.00–18.00 min; the mass range was set as 200–1000 Da; the intensity threshold and noise elimination level were set at 0 and 6, respectively; mass and retention time window was set to 0.02 and 0.20. Then, the data matrix of peak number (t_R -*m/z* pair), sample name and ion intensity were extracted and the missing values or zero values of MS variables higher than 80% were deleted. Finally, all the data were input to the SIMCA-P software to perform PCA and OPLS-DA. The drug-containing groups and the control group were subjected to the OPLS-DA to generate S-plot. As a result, the interesting ions which were present in the raw and honey-processed *Astragalus* groups (*Astragalus* groups) and absent in the control group were extracted with the aid of the variable importance of projection (VIP) and S-plot. These ions were identified according to their elemental compositions, MS/MS fragment mass spectra, and databases. The data of raw and honey-processed *Astragalus* groups were compared according to the PCA, OPLS-DA and independent sample *t* test was performed with the ions with the $\text{VIP} > 1$ at a confidence level of $> 95\%$ ($p < 0.05$) in the SPSS Version 20.0 software (IBM Co., Armonk, NY, USA).

Results and Discussion

Strategies for Screening and Identification of the Metabolites of Water Extracts of Raw and Honey-Processed *Astragalus* in Rat Urine

We established the fingerprints of water extracts of raw and honey-processed *Astragalus* by UHPLC/ESI-Q-TOF-MS in

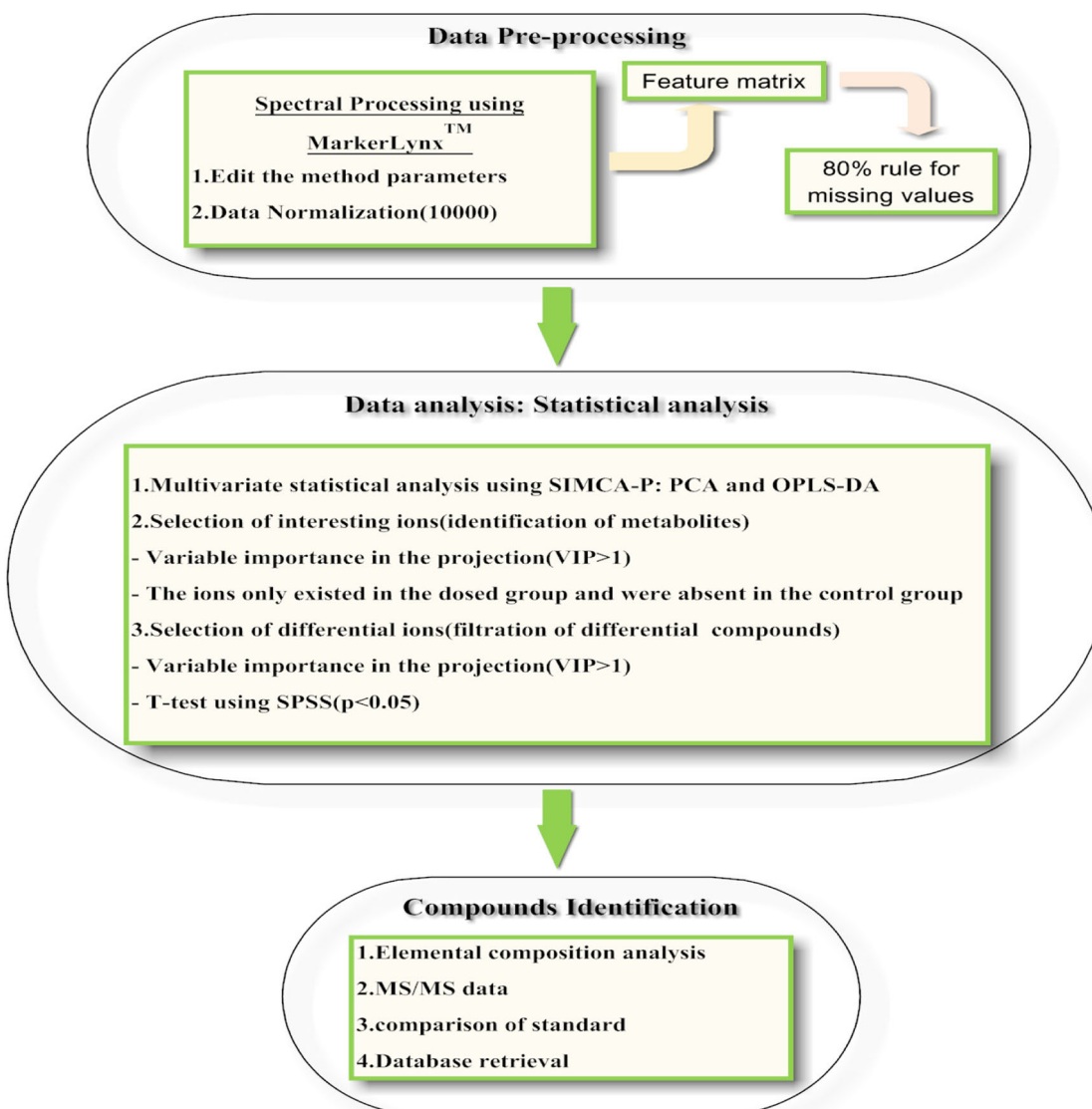


Figure 1. Summary of data pre-processing, data analysis, and compounds identification performed in this study

both positive and negative ions before analyzing drug-contained rat urine. The constituents and the discrepant compounds between them in vitro have been identified in our previous work [20]. In order to obtain a global view of rat urinary metabolite profiles, rat urine samples of raw and honey-processed *Astragalus* groups in 24 h were collected after a 3-day oral administration with the water extracts. The urine samples were treated with methanol and then analyzed and compared according to the established method. The detection of prototypes was performed based on the extracted ion chromatograms (EICs) function and the comparison between the fingerprints of water extracts of raw and honey-processed *Astragalus*.

Due to the high sensitivity of UHPLC/ESI-Q-TOF-MS, the substrate effect of complex biological matrices usually affects the metabolite identification. It is part of the defects of UHPLC/ESI-Q-TOF-MS in the determination of biological samples. Actually, decreasing the matrix effect helps to the identification

of metabolites, but it also affects the detection results. Therefore, we can eliminate matrix effects according to the processing methods. Principal component analysis (PCA) can be used to analyze the main influencing factors and simplify complicated problems [23]. Partial least squares-discriminant analysis (PLS-DA) is a supervised discriminant analysis method and especially suitable for small observations [24, 25]. Orthogonal partial least squares-discriminant analysis (OPLS-DA) eliminates the variables orthogonal to Y value on the basis of PLS-DA [26]. In addition, OPLS-DA is of better transparency and explanatory power than PLS-DA model and does not reduce the ability of model prediction [27]. As mentioned above, metabolites only existed in the *Astragalus* groups and the ions of metabolites were the most important variables contributing to the difference between the control group and *Astragalus* groups. The variable importance of projection (VIP) was used to quantify the contribution of each variable to classification. The larger VIP indicates the more significant contribution of

the variable [28]. In this paper, OPLS-DA was used to identify unknown and known metabolites. Firstly, the OPLS-DA was carried out with the blank urine and drug-contained urine. Then, the S-plot was obtained through OPLS-DA between the two groups. Ions were selected from VIP and trend plot, and the ions which only existed in the *Astragalus* groups and absent in the control group were considered as potential in vivo migration components.

Identification of Prototypes Base peak ion chromatograms of the control group, raw *Astragalus* group, and honey-processed *Astragalus* group obtained by UHPLC/ESI-Q-TOF-MS are shown in Figure 2. According to the extracted ion chromatograms (EICs) function, seven compounds were identified as parent compounds in drug-contained urine in comparison with the constituents in the water extracts of *Radix Astragali* and honey-processed *Astragalus*. Most of the parent compounds were identified based on the retention time and TOF-MS data (Table 1).

Seven prototypes, including isoflavones and their glycosides and astragaloside IV, were detected in *Radix Astragali* and honey-processed *Astragalus* by this method. Actually, only astragaloside IV was detected in rat urine, indicating that it was difficult for saponins to enter the circulation system or be metabolized by other means. Saponins were detected from rat bile after oral administration of alcohol extracts of a prescription containing *Radix Astragali* and both the types and contents of saponins were increased. It indicated that they were mainly excreted into bile [31]. In order to test whether the substances in rat urine affect the detection of the saponins, the astragaloside I was put into the blank urine and then prepared it by the same processing method. Interestingly, the results showed that astragaloside I was turned into isomers of astragaloside I, astragaloside II, and its isomers at 0 h (Figure 3a), and totally converted into astragaloside II, isomers of astragaloside II, and astragaloside IV after 48 h (Figure 3b). As shown in Figure 3c, the structures of astragaloside I, astragaloside II, and

astragaloside IV were different from the red frames and they were 2',3'-di-O-Ac- β -D-xyl, 2'-O-Ac- β -D-xyl and β -D-xyl, respectively. Finally, 2', 3'-di-O-Ac- β -D-xyl and 2'-O-Ac- β -D-xyl were turned into the β -D-xyl, suggesting that the conditions in rat urine were conducive to hydrolysis reactions. Astragaloside I and astragaloside II in rat urine are completely hydrolyzed into astragaloside IV because the urine was collected within 24 h in the study. Therefore, only astragaloside IV was detected. The results also suggested that the main active form of saponins was astragaloside IV. It was consistent with the quality requirements of *Radix Astragali* in Chinese Pharmacopoeia 2015.

Identification of Metabolites According to the strategy mentioned above, the OPLS-DA score plots of the control group and *Astragalus* groups were established to illustrate the relationship between these two groups in both positive and negative ion mode. As shown in Figure 4a, b, c, d, the control group and *Astragalus* groups have a clustered trend, indicating that components changed significantly in the control group and *Astragalus* groups. Loading S-plot of OPLS-DA (Figure 4e, f, g, h) clearly shows significant variables which can differentiate the two groups and each dot displays one of the variables from urine samples detected using UHPLC-Q-TOF-MS. The dots at the top or bottom ends of the "S" represent most significantly changed variables within the control and *Astragalus* groups. Those variables include the absorbed ingredients (prototype), metabolites, and endogenous metabolites. The prototype components and their metabolites were considered to be the variables which were present in the *Astragalus* groups and absent in the control group. In order to eliminate the disturbance from endogenous metabolites which were observed in both groups, prototype ingredients and their metabolites were extracted using the trend plots. t_R - m/z 3.01-623.1604, t_R - m/z 7.07-477.1431, t_R - m/z 9.43-243.1031 and t_R - m/z 4.06-658.2341 in the positive ion mode were taken as examples in Figure 5a, b, c, d which were extracted at the top

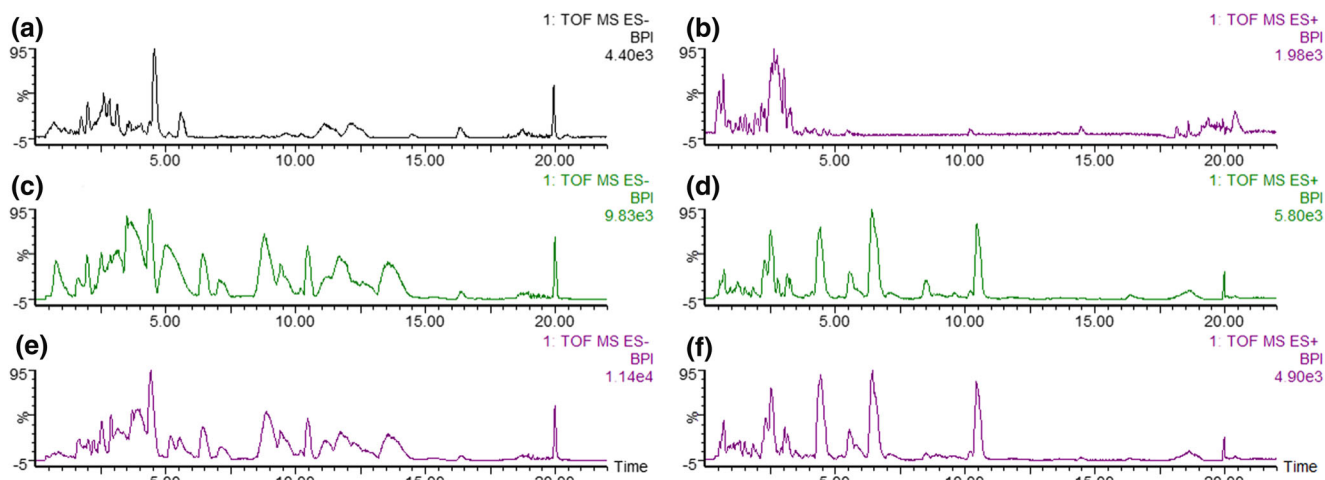


Figure 2. The BPI chromatograms of rat urine samples by UHPLC/ESI-Q-TOF-MS (a, b) control group BPI; (c, d) honey-processed *Astragalus* group BPI; (e, f) raw *Astragalus* group BPI; left, negative ion mode; right, positive ion mode)

Table 1. Mass Data for Identification of Parent Compounds in Rat Urine Samples After Oral Administration with Water Extracts of Raw and Honey-Processed *Astragalus*

| No. | t _R (min) | Adduct ion | Measured value (m/z) | Calculated value (m/z) | Error (mDa) | Fragments | Formulas | Identification |
|-----|----------------------|---------------------------------------|----------------------|------------------------|-------------|-------------------------------------|---|--|
| P1 | 3.46 ^a | [M+H] ⁺ | 447.1286 | 447.1291 | -0.5 | 285 | C ₂₂ H ₂₃ O ₁₀ | Calycosin-7-O-β-D-glucoside |
| P2 | 4.74 ^a | [M+H] ⁺ | 431.1355 | 431.1342 | 1.3 | 269,254,237,213 | C ₂₂ H ₂₃ O ₉ | Ononin |
| P3 | 6.56 ^a | [M-H] ⁻ | 283.0580 | 283.0606 | -2.6 | 268,239,212 | C ₁₆ H ₁₁ O ₅ | Calycosin |
| | | [M+H] ⁺ | 285.0742 | 285.0763 | -2.1 | 270,253,225,214,137,185 | C ₁₆ H ₁₃ O ₅ | |
| P4 | 10.46 ^a | [M-H] ⁻ | 267.0643 | 267.0657 | -1.4 | 252,223,195,135 | C ₁₆ H ₁₁ O ₄ | Formononetin |
| | | [M+H] ⁺ | 269.0795 | 269.0814 | -1.9 | 254,237,225,213,198,181,170,136 | C ₁₆ H ₁₃ O ₄ | |
| P5 | 10.75 | [M-H] ⁻ | 299.0905 | 299.0919 | -1.4 | 284,269,241 | C ₁₇ H ₁₅ O ₅ | |
| | | [M+H] ⁺ | 301.1065 | 301.1076 | -1.1 | 241,167,147,285,268, 191,178,163 | C ₁₇ H ₁₇ O ₅ | (6aR,11aR)-3-Hydroxy-9,10-dimethoxypteroctarpin [29] |
| P6 | 11.13 ^a | [M+CH ₃ COOH] ⁻ | 843.4773 | 843.4742 | 3.1 | - | C ₄₃ H ₇₁ O ₁₆ | Astragaloside IV |
| | | [M+H] ⁺ | 785.4649 | 785.4687 | -3.8 | 605,587,473,455,437,419 | C ₄₁ H ₆₉ O ₁₄ | |
| P7 | 11.16 | [M-H] ⁻ | 301.1071 | 301.1076 | -0.5 | 270,256,229,121 | C ₁₇ H ₁₇ O ₅ | |
| | | [M+H] ⁺ | 303.1227 | 303.1232 | -0.5 | 167,152,133,123,147 | C ₁₇ H ₁₉ O ₅ | 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan [30] |

^aConfirmed by comparison with reference substances

of red dots in the S-plot. The red dots in the S-plot were the ingredients at a high level in *Astragalus* groups and the VIP > 1 in OPLS-DA results. Finally, a total of 55 metabolites were detected in drug-contained urine with OPLS-DA. As shown in Table 2, 45 metabolites including 9 phase-I and 36 phase-II metabolites were identified and 10 unknown compounds were detected. The empirical molecular formula of metabolites was achieved by the calculation with the MS data in MassLynx™. Metabolites were identified by the empirical molecular formula and MS/MS data based on SciFinder and METLIN databases.

Phase-I metabolites (M12, M20, M22, M27, M28, M40, M44, M45, and M48) are mainly products of methylation, demethylation, hydroxylation, and dehydroxylation reactions in vivo and their MS/MS fragmentations are similar to the parent compounds. For example, the retention time of M22 was 5.58–5.60 min on UHPLC. It was shown in the form of [M+H]⁺ at *m/z* 255.0653 in the positive ion mass spectrum and in the form of [M-H]⁻ at *m/z* 253.0429 in the negative ion mode. Therefore, the molecular mass of M22 was inferred to be 254 Da. In the positive MS² spectrum of *m/z* 255 (Figure 6), fragment ions at *m/z* 237 [M+H-H₂O]⁺, *m/z* 227 [M+H-CO]⁺, *m/z* 209 [M+H-H₂O-CO]⁺, *m/z* 199 [M+H-2CO]⁺, and *m/z* 181 [M+H-H₂O-2CO]⁺ indicated the presence of hydroxyl and carbonyl groups. Fragment ion at *m/z* 137 was derived from the A-ring of isoflavone. These data were identical to those of daidzeins reported in the METLIN database. Therefore, M22 was identified unequivocally as daidzein. The possible fragmentation behaviors were shown in Figure 7a.

The molecular ion peak *m/z* M45 was found to be 301.0709 Da, which was only 16 Da higher than that of calycosin in positive ion mode. Its chemical formula was speculated to be C₁₆H₁₃O₆ according to the elemental composition function of MassLynx, suggesting that it might be a hydroxy derivative of calycosin. In the MS² spectrum of *m/z* 301 (Figure 6), which was similar to that of calycosin, fragment ions at *m/z* 286 and *m/z* 269 indicated a sequential loss of 15 and 32 Da and suggested the presence of a methyl and methoxyl. The *m/z* 153 was generated by Retro Diels-Alder fragmentation in the C-ring, indicating that there were two hydroxy groups in the A-ring and that the newly added hydroxyl group was linked to A-ring. Other three product ions at *m/z* 241 [M+H-CH₃OH-CO]⁺, *m/z* 229 [M-CH₃-2CO]⁺, and *m/z* 213 [M+H-CH₃OH-2CO]⁺ were also observed. Based on these data, M45 was tentatively identified as A-ring-monohydroxylated calycosin. The possible fragmentation behaviors are shown in Figure 7b. M27 was also identified as A-ring-monohydroxylated calycosin. Additional phase-I compounds were identified according to the above methods.

Phase-II metabolites (M4~M11, M14~M19, M23, M25, M26, M29~M31, M34~M39, M42, M43, M46, and M49~M55) are mainly glucuronide and sulfate conjugates and MS² spectrum always shows the loss of 176 and 80 Da. Then, the fragment ions of aglycones were used to identify what they were. The *m/z* 285 was obtained from the MS² spectrum of the M14 and M43 at *m/z* 461 and *m/z* 365 in the positive ion mass spectrum. The loss of 176 and 80 Da

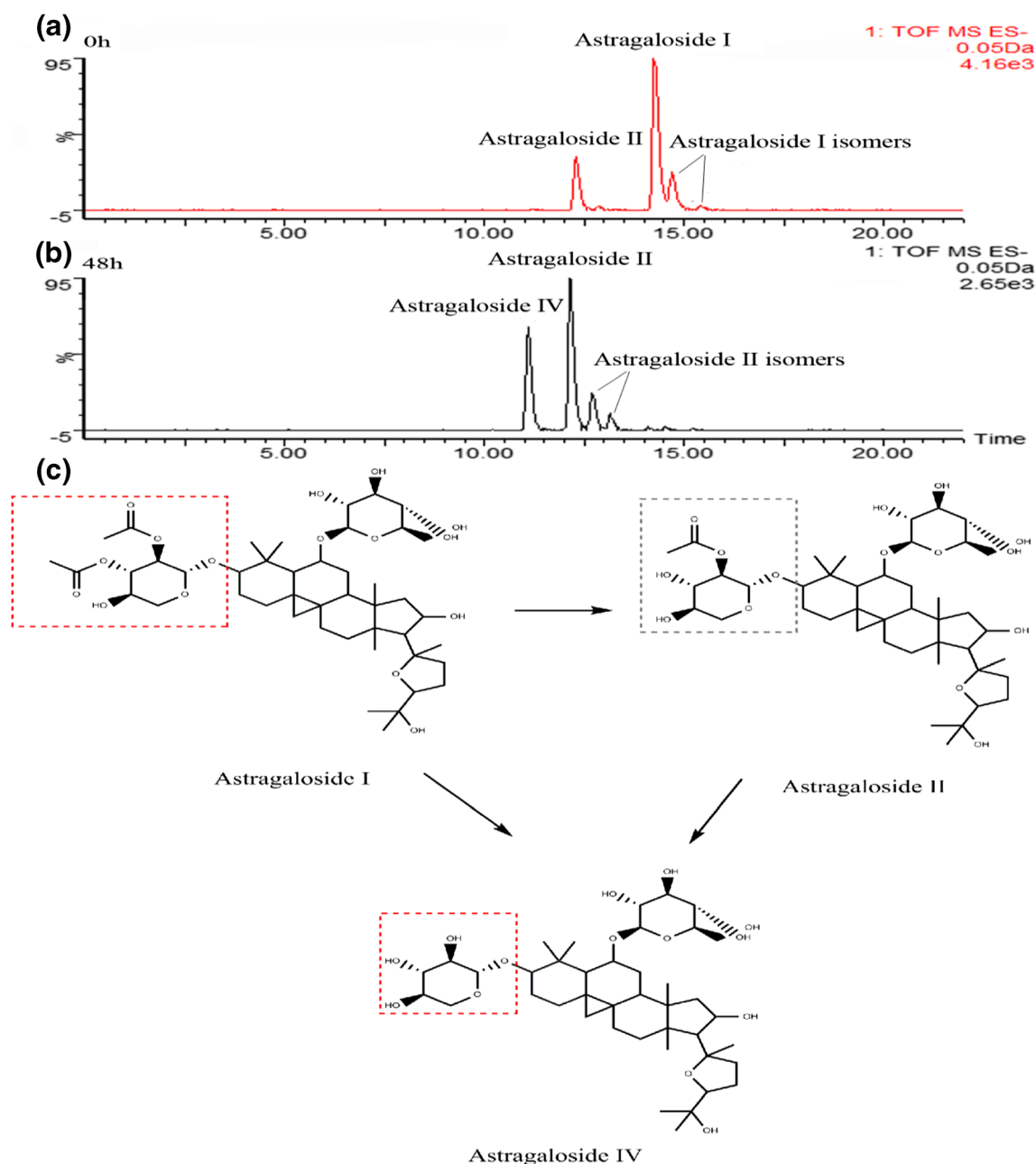


Figure 3. The EICs of the mixture samples which contains astragaloside I and blank urine **(a)** 0 h; **(b)** 48 h and the structures of astragaloside I, astragaloside II, and astragaloside IV **(c)**. Three substances were different from the red frames

indicated that they were glucuronide and sulfate conjugates. As shown in Figure 6, the fragment ions at m/z 270, m/z 253, and m/z 225 indicated a sequential loss of 15, 17, and 28 Da from aglycone ion m/z 285, suggesting the presence of methyl, hydroxyl, and carbonyl groups. The m/z 137 was generated by Retro Diels-Alder fragmentation in the C-ring at m/z 285. Other two productions at m/z 214[M+H-CH₃-2CO]⁺ and m/z 229[M+H-2CO]⁺ were obtained. Based on these data, m/z 285 was identified as calycosin according to the standard and reported data [11]. Therefore, M14 and M43 were identified as calycosin-7-O-glucuronide and calycosin-7-O-sulfate. The possible fragmentation behaviors are shown in Figure 7c. M46 was identified as calycosin-4'-O-sulfate. Other phase-II

metabolites were identified according to the method described above. It should be pointed out that the M15 only existed in the honey-processed *Astragalus* group.

The metabolites including phase-I and phase-II products in rat urine collected after the oral administration with the water extracts of raw and honey-processed *Astragalus* were systematically identified by UHPLC/ESI-Q-TOF-MS combined with OPLS-DA. Compared with the traditional detection methods of metabolites, this technique can eliminate the matrix effects and enhance the analysis efficiency in metabolism studies and is suitable to screen more low-level metabolites [36]. These metabolites identified from dosed urine are mainly phase-II products of isoflavones

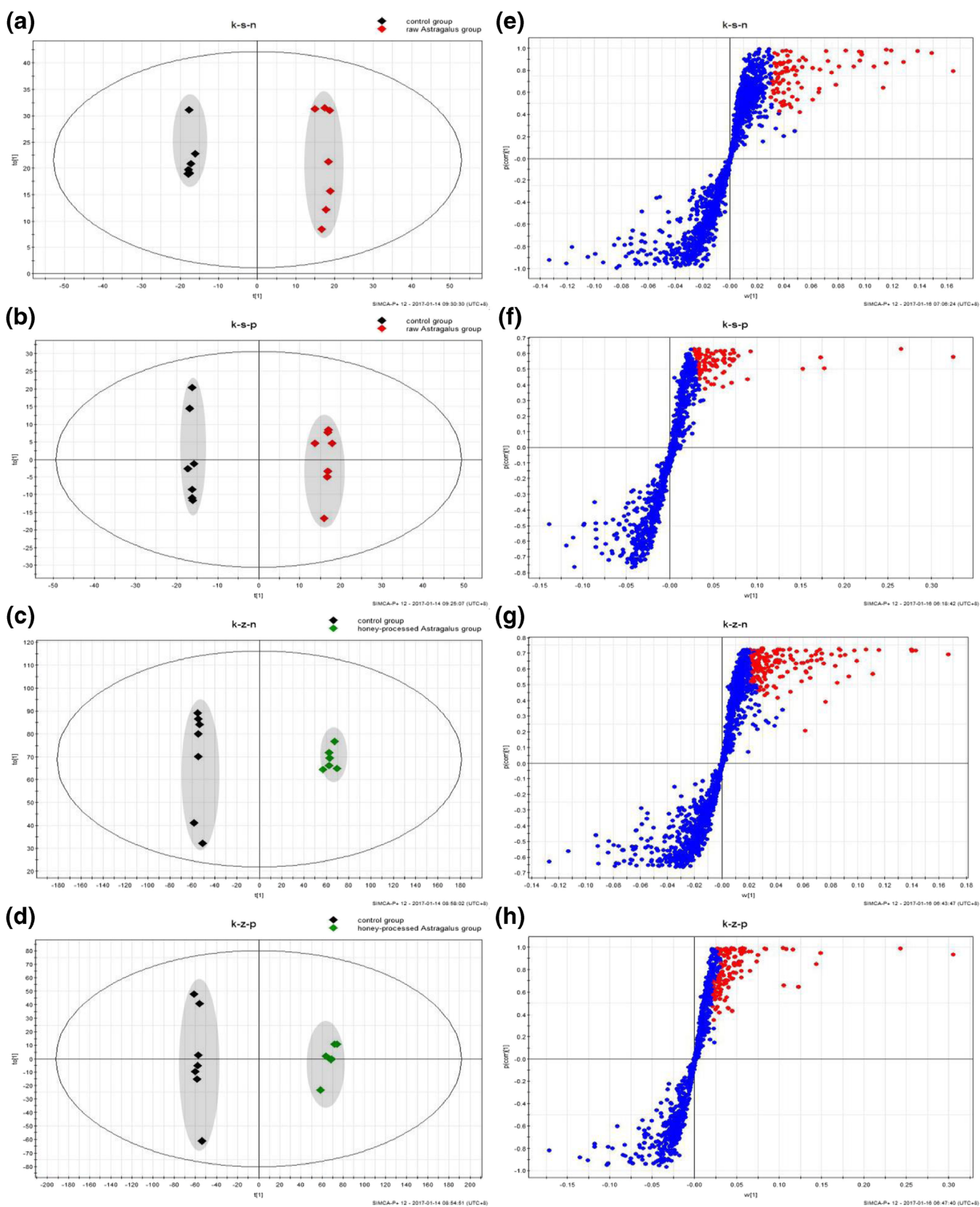


Figure 4. OPLS-DA score plots (left) and S-plots (right) for ESI⁻ (a, c, e, g) and ESI⁺ (b, d, f, h). Black: control group, red: raw Astragalus group, green: honey-processed Astragalus group in the OPLS-DA score plots

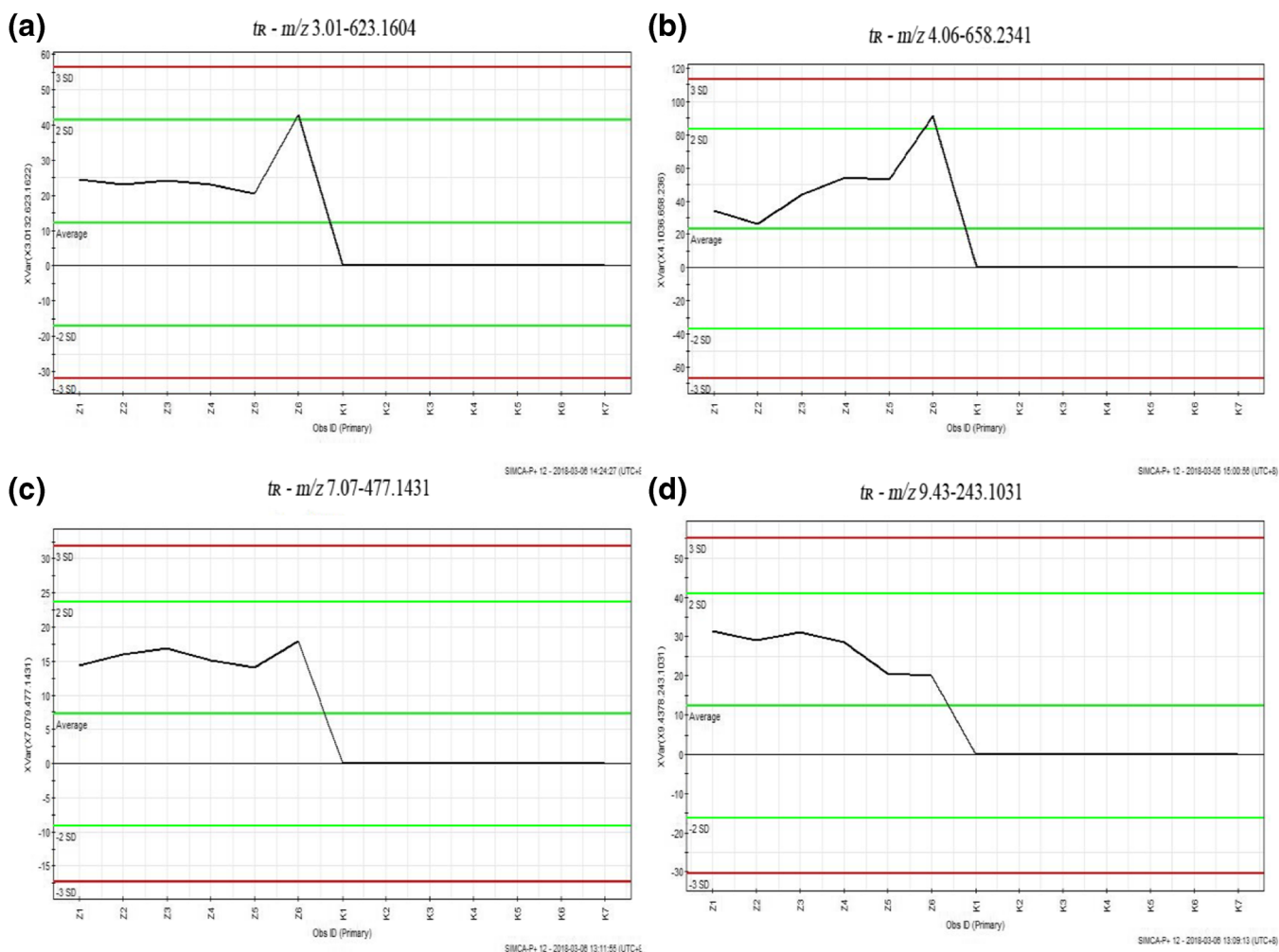


Figure 5. The trend plots of t_R - m/z 3.01–623.1604 **(a)**, t_R - m/z 4.06–658.2341 **(b)**, t_R - m/z 7.07–477.1431 **(c)**, and t_R - m/z 9.43–243.1031 **(d)** in the positive ion mode, between the control group(K1–K7) and honey-processed *Astragalus* group(Z1–Z6)

and possible metabolic pathways are shown in Figure 8. In the study, 11 metabolites in rat urine collected by metabolic cage within 24 h after oral administration of raw and honey-processed *Astragalus* were characterized for the first time: M6, M9, M12, M15, M16, M20, M26, M28, M30, M36, and M42. Additionally, to further confirm the identified phase-II metabolites, the β -glucuronidase was added into the drug-contained urine samples. The intensity of glucuronide conjugates (e.g., M31) decreased remarkably after enzymatic hydrolysis, whereas the aglycones (e.g., P7) increased correspondingly (Figure 9).

Multivariate Statistical Analysis of Raw and Honey-Processed *Astragalus*

PCA score plots (Figure 10a, b) and OPLS-DA score plots (Figure 10c, d) were acquired with the data of raw and honey-processed *Astragalus* groups in SIMCA-P software. PCA score plots showed clear separation from each other, suggesting that the two groups were different.

Therefore, the OPLS-DA was used to further explore the differences in drug metabolism after oral administration with the water extracts of raw and honey-processed *Astragalus*.

VIP reflects the importance of a variable in classification. The greater VIP indicates the more significant effects of the variable. We selected the variables with the $VIP > 1$ from OPLS-DA to perform the independent sample t test. The variables with $P < 0.05$ were regarded as potential biomarkers. According to the analogy method, 20 metabolites including 14 identified metabolites and 6 unidentified compounds were considered as different metabolites (Figure 11).

The efficacy in *Radix Astragali* was changed after honey-processing owing to the changes in chemical composition after honey-processing. The chemical composition before and after processing in vitro was compared [20]. In the study, the changes in the metabolism of raw and honey-processed *Astragalus* in vivo were explored in order to search for their effective substance basis. Then, 20 metabolites with differential compounds in raw and

Table 2. Mass Data for Identification of Metabolites in Rat Urine Samples After Oral Administration with Water Extracts of Raw and Honey-Processed *Astragalus*

| No. | t _R (min) | Adduct ion | Measured Value(m/z) | Calculated Value(m/z) | Error (mDa) | Fragments | Formulas | Identification | Metabolic type ^c |
|--------------------|----------------------|-----------------------------------|---------------------|-----------------------|-------------|---------------------------------|---|--|-----------------------------|
| M1 | 2.44 | [M-H] ⁻ | 239.0540 | 239.0556 | -1.6 | 149,159,131,117,115 | C ₁₁ H ₁₁ O ₆ | Unknown | - |
| M2 | 2.57 | [M+H] ⁺ | 267.1021 | 267.1010 | -1.1 | 203,174,144,123 | C ₁₇ H ₁₅ O ₃ | Unknown | - |
| M3 | 2.81 | [M-H] ⁻ | 295.1334 | 295.1291 | -4.3 | 124,223,253 | C ₁₉ H ₁₉ O ₃ | Unknown | - |
| M4 | 3.01 | [M+H] ⁺ | 297.1467 | 297.1455 | -3.6 | 279,255,239,192,134 | C ₁₉ H ₂₁ O ₃ | Calycosin-7-O-β-D-glucuronide-3'-O-glucuronide [32, 33] | II |
| M5 | 3.26 | [M+H] ⁺ | 621.1439 | 621.1456 | -1.7 | 459,283,175 | C ₂₈ H ₂₉ O ₁₆ | Daidzein-7-O-glucuronide [32] | II |
| M6 ^b | 3.43 | [M+H] ⁺ | 429.0800 | 429.0822 | -2.2 | 623,1612 | C ₂₈ H ₃₁ O ₁₆ | Double glucuronides conjugate of 2',3',4',7-tetrahydroxyisoflavan | II |
| M7 | 3.52 | [M+H] ⁺ | 431.0958 | 431.0978 | -2.0 | 253,175 | C ₂₁ H ₁₇ O ₁₀ | Hydroxylated calycosin-7-O-glucuronide | II |
| M8 | 3.73 | [M+H] ⁺ | 625.1417 | 625.1408 | -0.9 | 449,273,175 | C ₂₇ H ₂₉ O ₁₇ | Double glucuronides conjugate of 2',3',4',7-tetrahydroxyisoflavan | II |
| M9 ^b | 3.93 | [M+NH ₄] ⁺ | 644.1829 | 644.1827 | 0.2 | 451,275,167 | C ₂₇ H ₃₄ NO ₁₇ | Hydroxylated calycosin-7-O-glucuronide | II |
| M10 | 4.06 | [M+H] ⁺ | 477.1052 | 477.1033 | 1.9 | 301,269 | C ₂₂ H ₂₁ O ₁₂ | 4,7,4'-Trihydroxyisoflavan-7-O-glucuronide [29] | II |
| M11 | 4.09 | [M+H] ⁺ | 433.1119 | 433.1135 | -1.6 | 415,257,175,113 | C ₂₁ H ₂₁ O ₁₀ | Double glucuronides conjugate of 2',3',4',7-tetrahydroxyisoflavan | II |
| M12 ^b | 4.11 | [M+NH ₄] ⁺ | 452.1543 | 452.1557 | -1.4 | - | C ₂₁ H ₂₆ NO ₁₉ | 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan-7-O-β-D-glucosyl-2'-O-glucuronide [11] | II |
| M13 | 4.12 | [M+H] ⁺ | 625.1390 | 625.1408 | -1.5 | 449,273,175 | C ₂₇ H ₂₉ O ₁₇ | 7,3',4'-Trihydroxyisoflavanone glucuronide [33] | I |
| M14 | 4.19 | [M+H] ⁺ | 644.1852 | 644.1827 | 2.5 | 451,275,167 | C ₂₇ H ₃₄ NO ₁₇ | Unknown | II |
| M15 ^{a,b} | 4.25 | [M-H] ⁻ | 639.1901 | 639.1925 | -2.1 | 463,301,175,286 | C ₂₉ H ₃₅ O ₁₆ | Calycosin-7-O-glucuronide [33] | II |
| M16 ^b | 4.65 | [M+NH ₄] ⁺ | 658.2341 | 658.2347 | -0.6 | 479,465,303,443,429,289 | C ₂₉ H ₄₀ NO ₁₆ | Unknown | II |
| M17 | 5.03 | [M+H] ⁺ | 447.0954 | 447.0927 | 2.7 | 271,215,137 | C ₂₁ H ₁₉ O ₁₁ | 2'-Hydroxy-3',4',7-trimethoxyisoflavan | II |
| M18 | 5.27 | [M+H] ⁺ | 315.1246 | 315.1232 | 1.4 | 285,267,241 | C ₁₈ H ₁₉ O ₅ | Equol-7-O-glucuronide [32] | II |
| M19 | 5.44 | [M+H] ⁺ | 281.1236 | 281.1224 | -1.2 | 162,136,120 | C ₁₁ H ₂₁ O ₈ | Equol-4'-O-glucuronide [32] | II |
| M20 ^b | 5.38 | [M+H] ⁺ | 459.0861 | 459.0927 | -6.6 | 283,268,175,137 | C ₂₂ H ₁₉ O ₁₁ | Formononetin 7-O-glucuronide [32] | II |
| M21 | 5.39 | [M+H] ⁺ | 461.1059 | 461.1084 | -2.5 | 285,270,253,243,227,213,137,197 | C ₂₂ H ₂₁ O ₁₁ | 7-Hydroxy-2',3',4'-trimethoxyisoflavan | I |
| M22 | 5.58 | [M+H] ⁺ | 449.1073 | 449.1084 | -1.1 | 273,253,241,175,151 | C ₂₁ H ₂₁ O ₁₁ | Unknown | II |
| M23 | 5.63 | [M+H] ⁺ | 625.1414 | 625.1405 | 0.9 | 449,273,175 | C ₂₇ H ₂₉ O ₁₇ | Double glucuronides conjugate of 2',3',4',7-Tetrahydroxyisoflavan | II |
| M24 | 5.73 | [M+H] ⁺ | 644.1853 | 644.1827 | 2.6 | 451,275,167 | C ₂₇ H ₃₄ NO ₁₇ | Equol-7-O-glucuronide [32] | II |
| M25 | 5.99 | [M+H] ⁺ | 417.1153 | 417.1186 | -3.3 | 241,175 | C ₂₁ H ₂₁ O ₉ | Formononetin 7-O-glucuronide [32] | II |
| M26 ^b | 6.45 | [M+H] ⁺ | 419.1355 | 419.1342 | 1.3 | 243 | C ₂₁ H ₂₃ O ₉ | 7-Hydroxy-2',3',4'-trimethoxyisoflavan | I |
| M27 | 6.42 | [M+H] ⁺ | 417.1174 | 417.1186 | -1.2 | 241,175 | C ₂₁ H ₂₁ O ₉ | Unknown | II |
| M28 ^b | 6.80 | [M+H] ⁺ | 419.1360 | 419.1342 | 1.8 | 243 | C ₂₁ H ₂₃ O ₉ | Daidzein [11] | I |
| M29 | 7.07 | [M+H] ⁺ | 443.0977 | 443.0978 | -0.1 | 267 | C ₂₂ H ₁₉ O ₁₀ | 7-Hydroxy-2',3',4'-trimethoxyisoflavane-7-O-sulfate | II |
| M30 ^b | 7.59 | [M+H] ⁺ | 445.1107 | 445.1135 | -2.8 | 254,237,213 | C ₂₂ H ₂₁ O ₁₀ | Unknown | II |
| M31 | 7.67 | [M+H] ⁺ | 315.1235 | 315.1232 | 0.3 | 297,267,241,285 | C ₁₈ H ₁₉ O ₅ | Hydroxylated calycosin-7-O-β-D-glucuronide [32] | II |
| M32 | 8.49 | [M+H] ⁺ | 299.1283 | 299.1299 | 1.6 | 281,263,235,145,133,107 | C ₁₈ H ₁₉ O ₄ | 2',3',4',7-Tetrahydroxyisoflavan glucuronide | II |
| | | | 253.0492 | 253.0501 | -0.9 | 225,209,197,180,135 | C ₁₅ H ₉ O ₄ | A-ring-monohydroxylated calycosin [33] | I |
| | | | 255.0636 | 255.0657 | -2.1 | 237,227,209,199,181,137 | C ₁₅ H ₁₁ O ₄ | Demethylated 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan | I |
| | | | 395.0786 | 395.0801 | -1.5 | 315,241,199 | C ₁₈ H ₁₉ O ₈ S | (6aR,11aR)-3-Hydroxy-9,10-dimethoxypteroocarpan-3-O-glucuronide [32] | II |
| | | | 301.1440 | 301.1473 | 3.3 | 271,253,241,146,133,106 | C ₁₈ H ₂₁ O ₄ | 2',3',4',7-Tetrahydroxyisoflavan-7-O-β-D-glucoside sulfate | II |
| | | | 475.0846 | 475.0877 | -3.1 | 299 | C ₂₂ H ₁₉ O ₁₂ | 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan-7-O-glucuronide [32] | II |
| | | | 477.1083 | 477.1033 | 5.0 | 301,286,269,133 | C ₂₂ H ₂₁ O ₁₂ | Unknown | II |
| | | | 449.1072 | 449.1084 | -1.2 | 273,121 | C ₂₁ H ₂₁ O ₁₁ | Hydroxylated calycosin-7-O-β-D-glucuronide [32] | II |
| | | | 299.0566 | 299.0556 | 1.0 | 284,268,239 | C ₁₆ H ₁₁ O ₆ | 2',3',4',7-Tetrahydroxyisoflavan glucuronide | II |
| | | | 301.0729 | 301.0712 | 1.7 | 286,269,241,229,213,153 | C ₁₆ H ₁₅ O ₆ | A-ring-monohydroxylated calycosin [33] | I |
| | | | 287.0932 | 287.0919 | 1.3 | 272,257,150,135,121 | C ₁₆ H ₁₅ O ₅ | Demethylated 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan | I |
| | | | 475.1213 | 475.1240 | -2.7 | 299,284,113 | C ₂₃ H ₂₃ O ₁₁ | (6aR,11aR)-3-Hydroxy-9,10-dimethoxypteroocarpan-3-O-glucuronide [32] | II |
| | | | 477.1431 | 477.1397 | 3.4 | 301,167,123 | C ₂₃ H ₂₅ O ₁₁ | 2',3',4',7-Tetrahydroxyisoflavan-7-O-β-D-glucoside sulfate | II |
| | | | 515.0878 | 515.0859 | 1.9 | 435,273,241,135 | C ₂₃ H ₂₅ O ₁₃ S | 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan-7-O-glucuronide [32] | II |
| | | | 477.1393 | 477.1397 | -0.4 | 301,286,175,113 | C ₂₃ H ₂₅ O ₁₁ | Unknown | II |
| | | | 479.1574 | 479.1553 | 2.1 | 303,167,149,123,181 | C ₂₃ H ₂₇ O ₁₁ | | |
| | | | 305.1865 | 305.1858 | -0.7 | - | C ₁₇ H ₂₅ N ₂ O ₃ | | |

Table 2 (continued)

| No. | t _R (min) | Adduct ion | Measured Value(m/z) | Calculated Value(m/z) | Error (mDa) | Fragments | Formulas | Identification | Metabolic type ^c |
|------------------|----------------------|--------------------|---------------------|-----------------------|-------------|-----------------------------|---|---|-----------------------------|
| M33 | 8.58 | [M+H] ⁺ | 307.2039 | 307.2032 | -0.7 | 247,182,164,122 | C ₁₇ H ₂₅ N ₂ O ₃ | Unknown | - |
| M34 | 8.71 | [M-H] ⁻ | 385.1499 | 385.1479 | -2.0 | 323,303,259 | C ₁₈ H ₂₅ O ₇ | Unknown | II |
| M35 | 8.85 | [M-H] ⁻ | 395.0794 | 395.0801 | -0.7 | 315,297,271,253,241,189,107 | C ₁₈ H ₁₆ O ₈ S | 2'-Hydroxy-3',4',7'-trimethoxyisoflavane-2'-O-sulfate | II |
| M36 ^b | 8.83 | [M-H] ⁻ | 397.0599 | 397.0593 | 0.6 | 317,297,271,253,241 | C ₁₇ H ₁₇ O ₉ S | Daidzein-7-O-sulfate [34] | II |
| M37 | 8.97 | [M-H] ⁻ | 349.0028 | 349.0018 | 1.0 | 269,252,239,223,197,137 | C ₁₃ H ₆ O ₈ S | A-ring hydroxylated 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan sulfate | II |
| M38 | 9.08 | [M-H] ⁻ | 333.0071 | 333.0069 | 0.2 | 255,227,209,199,181,137 | C ₁₃ H ₆ O ₇ S | 7,3',4'-Trihydroxyisoflavone sulfate | II |
| M39 | 9.08 | [M-H] ⁻ | 367.0474 | 367.0488 | 0.1 | 287,272,151,135,121 | C ₁₆ H ₁₅ O ₈ S | Daidzein-4'-O-sulfate [34] | II |
| M40 | 9.12 | [M+H] ⁺ | 271.0610 | 271.0606 | 0.4 | 215,137,225,197 | C ₁₃ H ₁₁ O ₅ | Demethylated-7,2'-Dihydroxy-3',4'-dimethoxyisoflavan sulfate [34] | II |
| M41 | 9.24 | [M-H] ⁻ | 297.1127 | 297.1130 | -2.7 | 253,189,145,121,107 | C ₁₈ H ₁₇ O ₄ | 7,3',4'-trihydroxyisoflavone [35] | I |
| M42 ^b | 9.25 | [M-H] ⁻ | 299.1283 | 299.1303 | 1.7 | 245,235,133,107 | C ₁₈ H ₁₆ O ₄ | Unknown | - |
| M43 | 9.41 | [M-H] ⁻ | 397.0598 | 397.0593 | 0.5 | 317,302,151 | C ₁₇ H ₁₇ O ₉ S | A-ring hydroxylated 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan sulfate | II |
| M44 | 9.43 | [M+H] ⁺ | 363.0167 | 363.0175 | -0.8 | 283,268 | C ₁₆ H ₁₁ O ₈ S | Calycosin-7-O-sulfate [34] | II |
| M45 | 9.61 | [M-H] ⁻ | 365.0330 | 365.0331 | -0.1 | 285,270,253,225,214,137 | C ₁₆ H ₁₃ O ₈ S | Equol [36] | I |
| M46 | 9.75 | [M-H] ⁻ | 241.0847 | 241.0865 | -1.8 | 223,121 | C ₁₅ H ₁₅ O ₃ | A-ring-monohydroxylated calycosin [33] | I |
| M47 | 9.92 | [M+H] ⁺ | 299.0565 | 299.0556 | 0.9 | 284, 268, 239 | C ₁₆ H ₁₁ O ₆ | A-ring-monohydroxylated calycosin [33] | I |
| M48 | 10.75 | [M+H] ⁺ | 341.1448 | 341.1442 | -0.6 | 285,270,253,225,214,137 | C ₁₆ H ₁₃ O ₈ S | Calycosin-4'-O- sulfate [34] | II |
| M49 | 11.15 | [M-H] ⁻ | 337.0348 | 337.0382 | -3.4 | 261,243,227,215,199,133 | C ₁₅ H ₂₅ O ₁₀ | Unknown | - |
| M50 | 11.45 | [M-H] ⁻ | 321.0433 | 321.0433 | 0.0 | 284,256,243,227,213,197,137 | C ₁₇ H ₁₅ O ₅ | 3'-Monomethylated-calycosin | I |
| M51 | 11.79 | [M-H] ⁻ | 337.0373 | 337.0382 | -0.9 | 257,137,151,121 | C ₁₅ H ₁₆ O ₆ S | 4,7,4'-Trihydroxyisoflavane sulfate [34] | II |
| M52 | 12.01 | [M-H] ⁻ | 321.0432 | 321.0433 | -0.1 | 241,121,223 | C ₁₅ H ₁₅ O ₆ S | Equol-4'-O- sulfate | II |
| M53 | 13.13 | [M-H] ⁻ | 353.0310 | 353.0331 | -2.1 | 273,255,151,135 | C ₁₅ H ₁₃ O ₈ S | (3R)-2',3',4',7'-Tetrahydroxyisoflavan-sulfate [32] | II |
| M54 | 13.55 | [M-H] ⁻ | 353.0333 | 353.0331 | 0.2 | 273,255,151,135 | C ₁₅ H ₁₃ O ₈ S | (3R)-2',3',4',7'-Tetrahydroxyisoflavan-sulfate [32] | II |
| M55 | 14.20 | [M-H] ⁻ | 367.0506 | 367.0488 | 1.8 | 287,272,151,135,121 | C ₁₆ H ₁₅ O ₈ S | Demethylated 7,2'-Dihydroxy-3',4'-dimethoxyisoflavan sulfate [34] | II |

- Data unavailable

*Only detected in the honey-processed group

^bDetected for the first time^cI, phase-I metabolites; II, phase-II metabolites

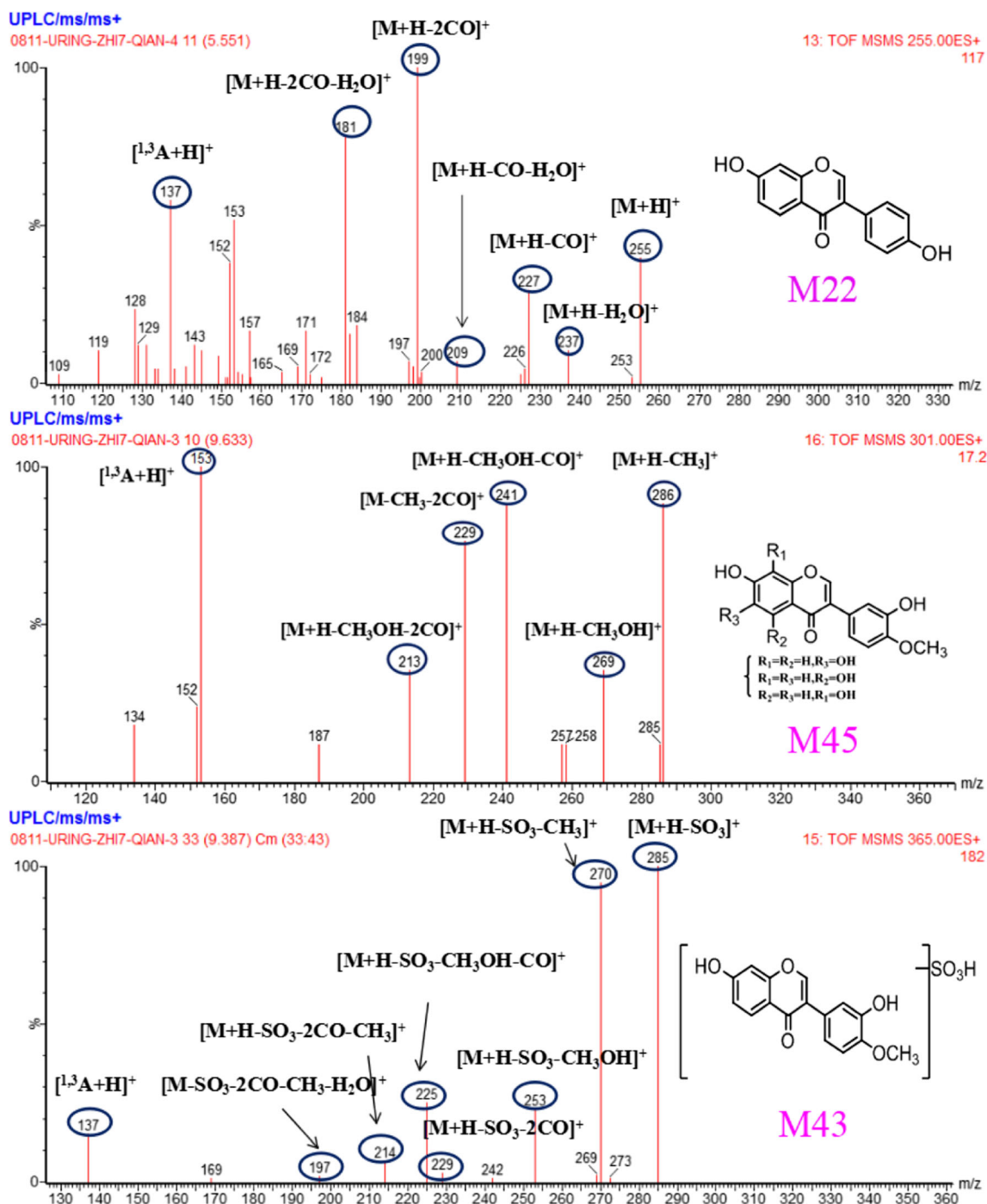


Figure 6. The MS² spectrum of M22, M45, and M43

honey-processed *Astragalus* groups were found. The 14 identified compounds were calycosin (P3), formononetin (P4), (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpa (P6), calycosin-7-O-β-D-glucuronide (M14), daidzein (M22), demethylated 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (M28), demethylated-7,2'-dihydroxy-3',4'-dimethoxyisoflavan sulfate (M39), daidzein sulfate (M35, M38), equol (M44), calycosin sulfate (M43, M46), and 3'-monomethylated-calycosin (M48), and 6 compounds were unknown. Among these differential

metabolites, the levels of M2, M3, M24, M28, M32, M41, and M44 in raw *Astragalus* group were significantly lower than those in the honey-processed *Astragalus* group and the levels of P3, P4, P5, M1, M14, M22, M35, M38, M39, M43, M46, and M48 in raw *Astragalus* group were significantly higher than those in the honey-processed *Astragalus* group. Moreover, M15 was only detected in the honey-processed *Astragalus* group, and P3, P4, and P5 were the prototypes which had the most potential as active ingredients. It is well

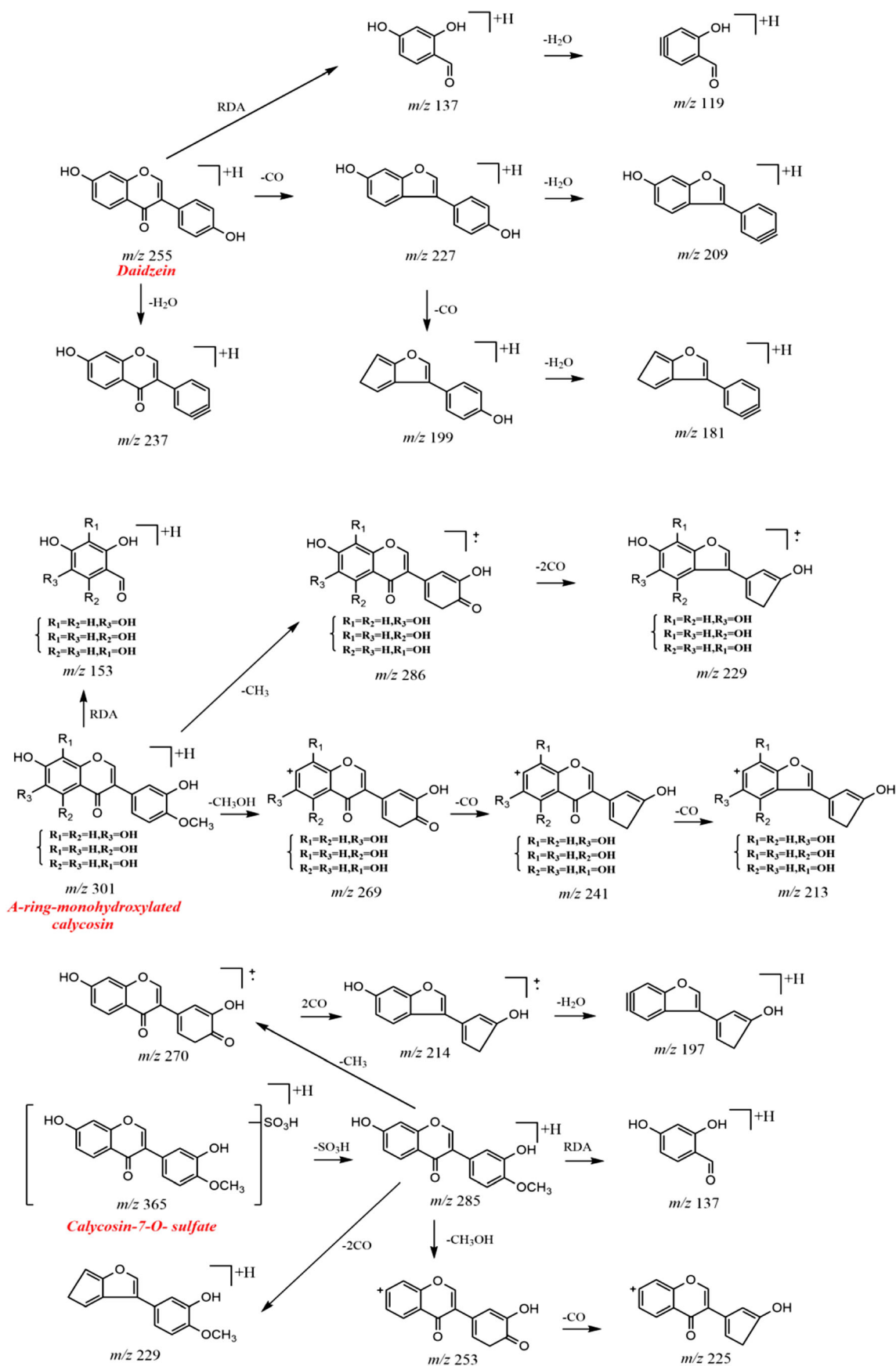


Figure 7. The typical fragmentation behaviors of M22 (a), M45 (b), and M43 (c)

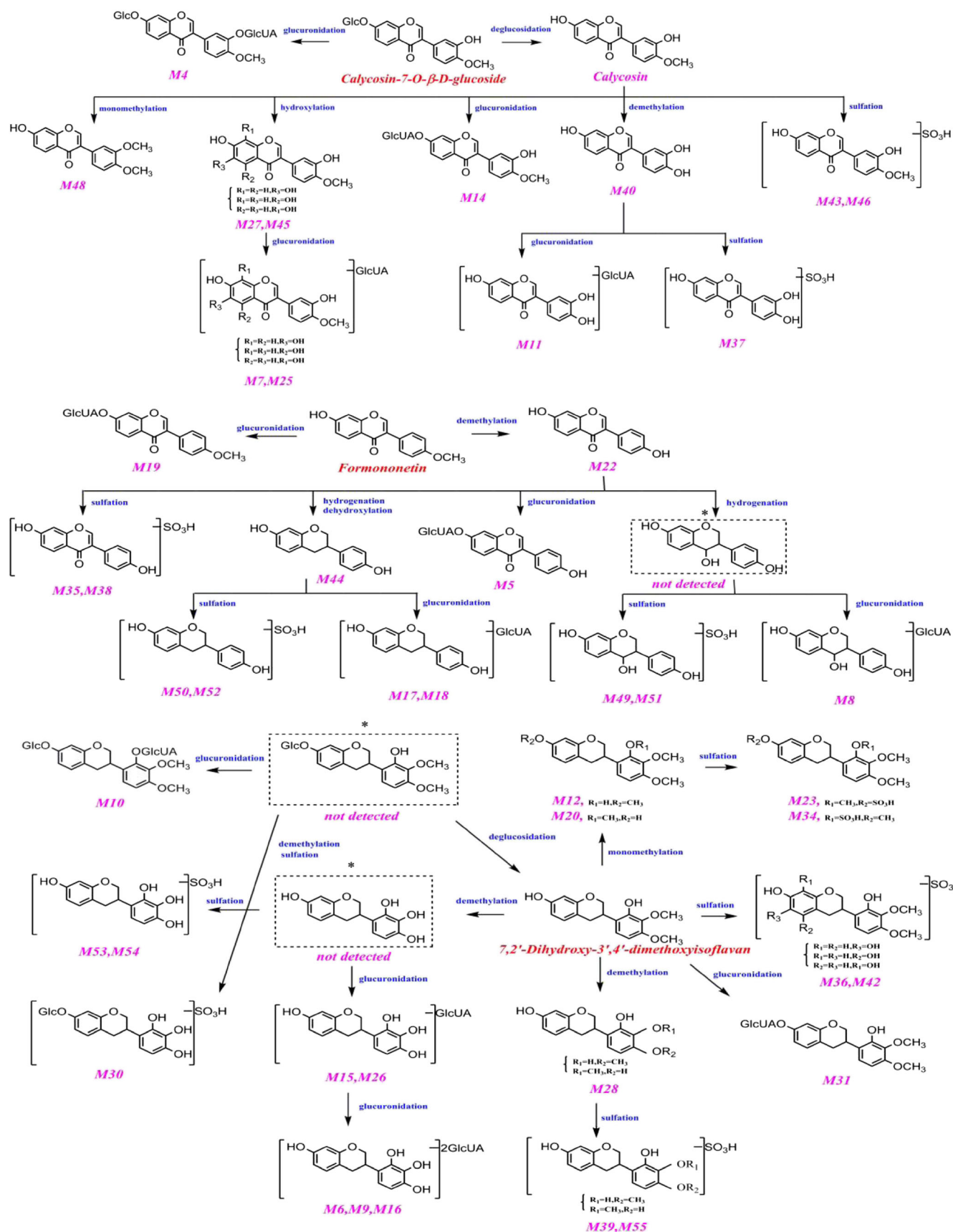


Figure 8. Chemical structures of isoflavonoid-related metabolites in rat urine and proposed major metabolic pathways of isoflavonoids. Compounds marked by asterisks are not detected

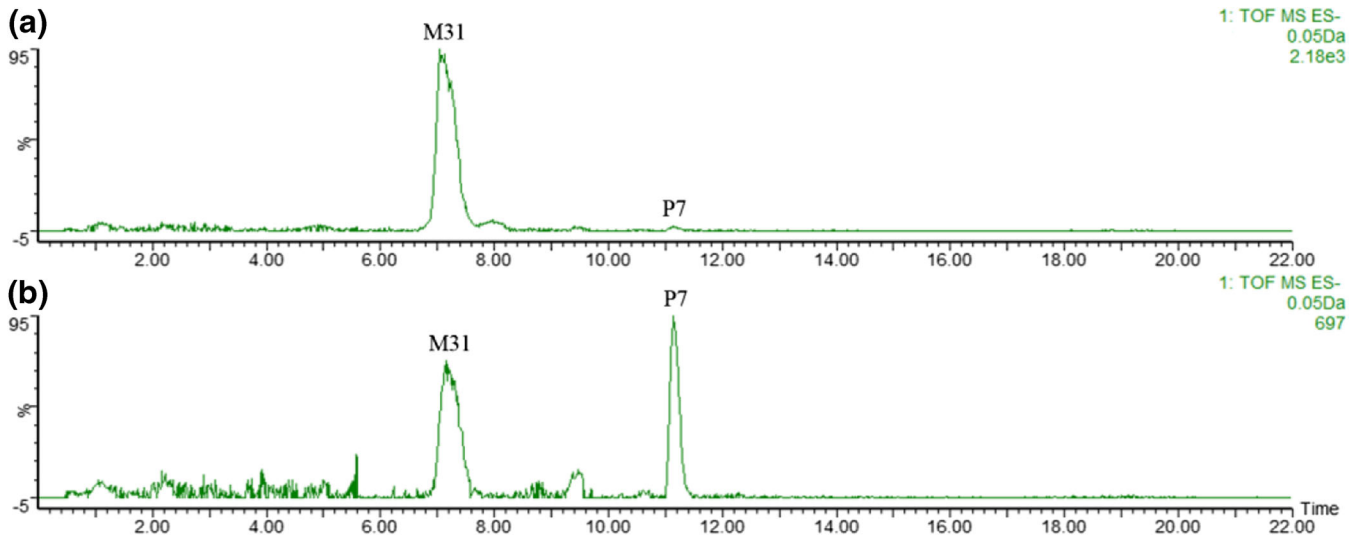


Figure 9. EICs of drug-containing urine sample by UHPLC/ESI-Q-TOF/MS in negative ion mode. (a) Before enzymatic hydrolysis; (b) after enzymatic hydrolysis

known that a drug has a dose-effect relationship. Therefore, the differential compounds in vivo are more

important for understanding the differential therapeutic effect between raw and honey-processed *Astragalus*.

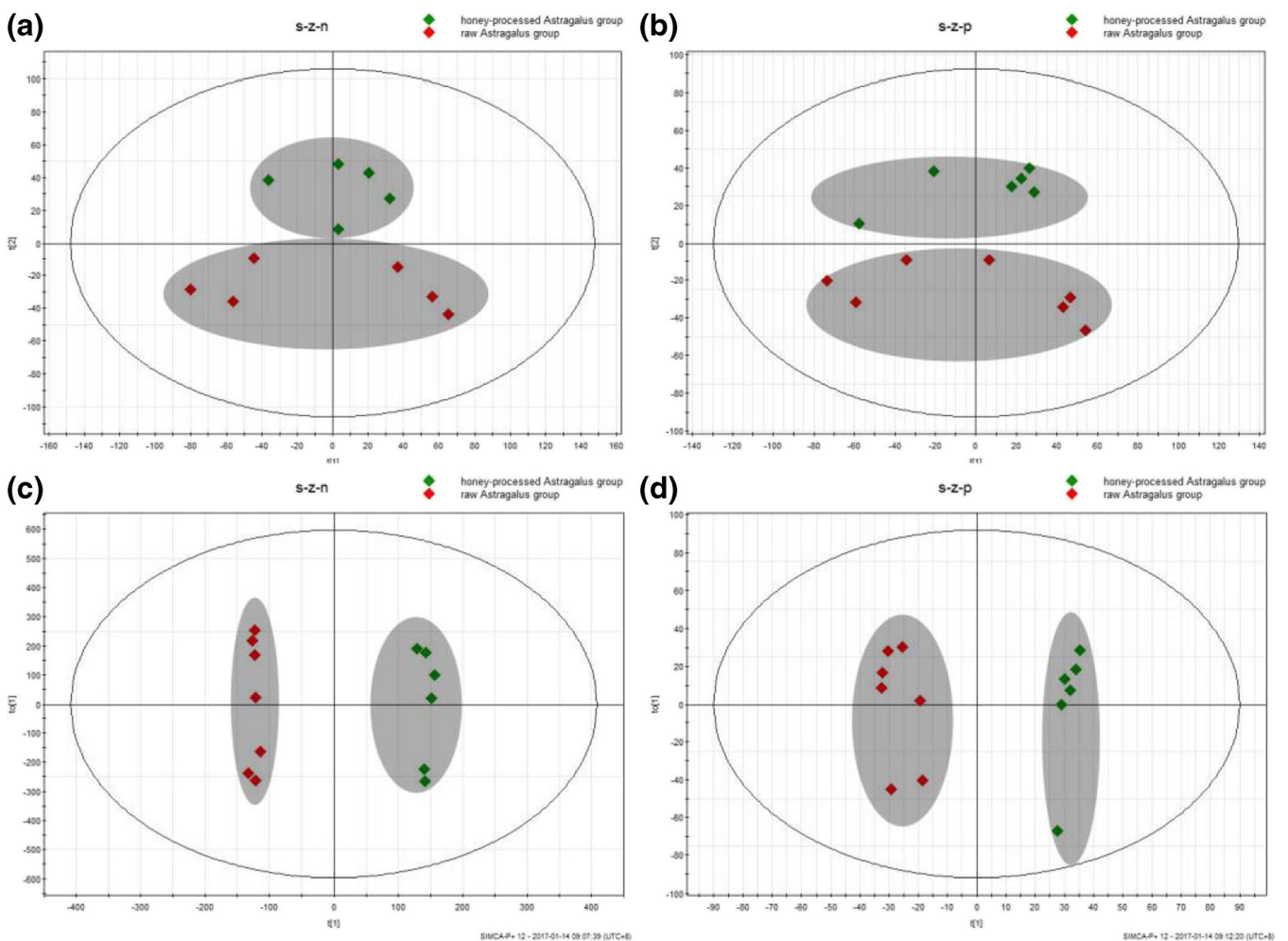


Figure 10. PCA (top) and OPLS-DA (bottom) score plots of the raw and honey-processed *Astragalus* groups for ESI⁻ (a, c) and ESI⁺ (b, d). Red: raw *Astragalus* group; green: honey-processed *Astragalus* group

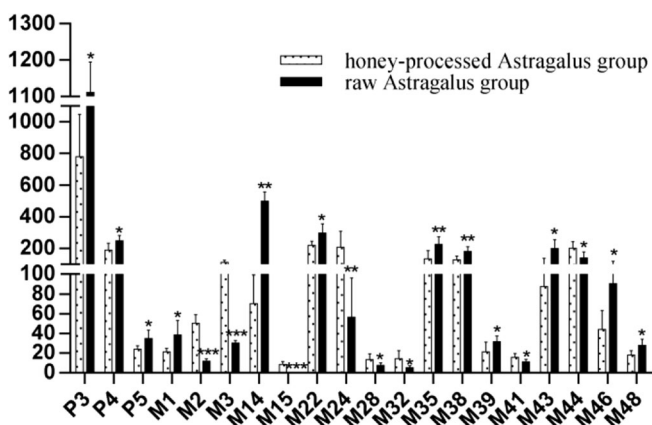


Figure 11. The levels of differences of 20 compounds between honey-processed *Astragalus* group and raw *Astragalus* group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; the y-axis represents the peak intensity)

Conclusions

In the study, UHPLC/ESI-Q-TOF-MS combined with multivariate statistical analysis was used to characterize the metabolites in rat urine after oral administration with the water extracts of raw and honey-processed *Astragalus*. In the metabolism of *Radix Astragali* and its honey-processed product in rat urine, 55 metabolites and 7 parent compounds were detected and 11 metabolites were identified for the first time. Simultaneously, 20 compounds in different levels were found in the raw and honey-processed *Astragalus* groups and 1 ingredient only existed in the honey-processed *Astragalus* group. These results might be helpful for investigating the bioactive mechanism of the differential therapeutic effects of raw and honey-processed *Astragalus*. Additionally, detection of urinary metabolites provides a study basis for serum pharmacochimistry of the raw and honey-processed *Astragalus* and the validated method provides an effective and exhaustive tool for screening the various metabolites of Chinese medicines.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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