Chemical Profiling of Wen-Dan Decoction by UPLC/Q-TOF-MS/MS and Variation Analysis between Different Processes Based on Chemometric

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Research

Keywords: chemical profiling, variation analysis, chemometric, preparation processes, Wen-Dan decoction, classic prescription

Posted Date: April 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-21340/v1

Chemical Profiling of Wen-Dan Decoction by UPLC/Q-TOF-MS/MS and Variation Analysis between Different Processes Based on Chemometric

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Abstract

Background: Wen-Dan decoction (WDD) is a traditional Chinese medical prescription composed of six herbs for the treatment of neurological diseases. The research of classic prescription (CP) is the hotspot in China which aims to develop the ancient and highly effective prescriptions into modern formulations under the condition of ancient dosage. In order to clarify the effects of ancient and modern techniques on WDD in the research of CP, the compounds in WDD should be systematically studied first, and the issue whether there are significant differences on components even efficacy between different preparation processes has to be explored.

Methods: In this study, ultra performance liquid chromatography coupled with

quadrupole time-of-flight mass spectrometry was employed to analyze the components of WDD rapidly in both positive and negative ion mode. The analysis methods of chemometric, such as partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA), was innovatively adopted to compare three typical processes (ancient decoction process, modern decoction process and modern alcohol precipitation process).

Results: The 121 compounds were identified in WDD, including 23 organic acids, 69 flavonoids, 4 amino acids, 5 phenylpropanoids, 6 triterpenoid saponins and 14 other components, and most of them were from Zhishi (Immature orange fruit), Chenpi (Dried tangerine peel) and Gancao (Liquorice root). Additionally, significant differences between three processes were proved in this study, and 24 components were considered to be related to the differences between ancient and modern technology, as well as 18 compounds could be affected in the process of alcohol precipitation. The relative content of compounds in modern decoction process was higher than that in ancient decoction process, indicating the extraction efficiency of modern technology was greater than that of ancient process.

Conclusion: The chemical profiling of WDD provided the bases for the study of active components and quality control. The obvious differences among three processes suggested that the content of active ingredients in the study of CP should be strictly controlled to ensure the transfer of significant efficacy from ancient prescriptions to modern formulations.

Keywords: chemical profiling; variation analysis; chemometric; preparation processes;

Wen-Dan decoction; classic prescription

Background

Wen-Dan decoction (WDD), recorded in the *Valuable Prescriptions for Emergency* written by Sun Simiao in tang dynasty, is composed of Banxia (Pinellia tuber), Zhishi (Immature orange fruit), Zhuru (Bamboo shavings), Chenpi (Dried tangerine peel), Gancao (Liquorice root) and Shengjiang (Ginger). WDD is a classic prescription (CP) for the treatment of phlegm, a disease related to nerves and emotions in traditional Chinese medicine (TCM) [1-2]. Obvious therapeutic effects of WDD on depression, insomnia, schizophrenia, alzheimer's disease and other neurological diseases have been proved by the modern clinical studies [3-6]. At present, the study of the compounds in WDD is not clear, which makes it difficult to determine the active components. However, it has been found that there are lots of flavonoids in Zhishi, Chenpi and Gancao as well as many organic acids in Banxia and Zhuru [7-10], which could provide references for the chemical profiling of WDD.

In recent years, a series of documents on the research and registration of CP has successively issued to support the development of TCM in China [11]. Due to the policy of exemption from clinical trials of CP, its development has attracted great attention from the researchers [12]. The purpose of CP research is to develop the ancient and highly effective prescriptions into modern formulations under the condition of following the ancient dosage. However, there are great differences between ancient and modern, such as dosage form, technical equipment and social environment. The

problem whether modern formulations of CP can still maintain its active components and significant efficacy remains to be explored. Therefore, the influences of different preparation processes in ancient and modern should be the focus of attention in the research of CP.

Generally speaking, due to the limited equipment, shorter time and few times of decoction, ancient decoction process (ADP) was inefficient, and the effective components were less dissolved. Modern decoction process (MDP) is more likely to optimize the decoction conditions to obtain the appropriate extraction efficiency. Modern alcohol precipitation process (MAPP) is often used to remove macromolecular substances (such as sugar, starch, protein) to reduce the oral dose. These three processes are the typical preparation methods of TCM prescription, which could greatly affect the type and content of ingredients.

With the advantages of high resolution, good sensitivity, short cycle and wide scanning range, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS/MS) is widely used in studying the compositions of TCM [13,14]. Additionally, the analysis methods of chemometric, such as partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA), can be adopted to analyze the differences between two groups of multiple variables [15-16]. In recent years, the combination of mass spectrometry and chemometric provides an analytical basis for the quality study of TCM from different regions or varieties [17-18].

This study analyzed the chemical constituents systematically to lay a foundation

for the further study about the identification of active components in WDD. In addition, by comparing three typical preparation processes, this paper purposed to investigate whether there were some significant differences between ancient and modern technology, and prepared for the problem whether different processes could affect the significant efficacy of WDD. It would promote the development of WDD traditional classic preparation into modern preparation and provide the reference for the study of WDD spectrum-effect correlation.

Methods

Materials and reagents

Fifteen batches samples of each herb were collected from three different regions of China (Additional file 1), and all of them were compatible with the validation test of Chinese Pharmacopoeia 2015 version. Reference substances were purchased from National Institutes of Food and Drug Control (Beijing, China) and Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China), the purity was higher than 98% by HPLC-UV analysis. LC-MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade. Ultra-high purity water was prepared by a Milli-Q Synthesis 108 water purification system (Bedford, MA, USA).

Preparation of WDD freeze-dried powder based on three processes

The 15 batches of each herb were randomly combined into 15 batches of WDD by

Excel to ensure the stability, reliability and representativeness. All sample were ensured to be used. Then, 15 batches of WDD were successively prepared based on the result of random combination and the follow processes.

Refer to the preparation process recorded in the *Valuable Prescriptions for Emergency* written by Sun Simiao in tang dynasty, 31.25 g Banxia, 31.25 g Zhuru, 31.25 g Zhishi, 46.86 g Chenpi, 62.50 g Shengjiang and 15.63 g Gancao were crush into coarse particles and put in a decoction pot with 1600 ml deionized water. The formulation was boiled at 500 W and evaporated to 400 mL at 200 W. The decoction was filtered with six layers of gauze, condensed in a rotary evaporator and converted into ADP-WDD freeze-dried powder in the Labconco Freeze Dry System.

The same 15 batches of WDD raw materials without smashing were immersed respectively in 8 times of water for 30 min, boiled for 2 h and continuously 3 times to obtain the MDP decoction. The decoction was filtered, condensed and dried in the same way of ADP to obtain the MDP-WDD freeze-dried powder.

The same 15 batches of MDP decoction were prepared. The decoction was condensed respectively and then mixed with ethanol until the alcoholicity reached 80%. After set at 4 ℃ for 12 h, the decoction was layered. The lower sediment was filtered out and the upper solution was condensed and dried in the same way to obtain the MAPP-WDD freeze-dried powder.

Preparation of sample solutions and standard solution

The WDD freeze-dried powder that equivalent to 2.0 g original herb was weighed

accurately. It was added to a 100 mL conical flask, extracted ultrasonically with 50 mL 50% ethanol for 45 min, centrifuged at 12000 rpm/min for 10 min and filtered through a 0.45 μm polytetrafluoroethylene filter to obtain the sample solutions. All 15 batches of sample solutions of ADP, MDP and MAPP were mixed in equal quantities before the chemical profiling by UPLC-Q-TOF-MS/MS. The reference substances were precisely weighed and prepared into mixed standard solution with methanol.

Conditions for liquid chromatography

A Shimadzu Controller CBM20Alite coupled with a quaternary pump, autosampler, and vacuum degasser (Shimadzu, Japan) was employed in this study. Chromatographic peak separation was performed on a Thermo Hyperall GOLD-C18 column (2.1mm×100mm, 1.8μm) with the column temperature of 40 °C. A gradient elution system composed of acetonitrile (A) and 0.1% formic acid (B) was used as follows: 0~2 min, 5% A; 2~30 min, 5% ~40% A; 30~32 min, 40%~75% A; 32~35 min, 75% A; 35~37 min, 75%~5% A; 37~40 min, 5% A. The flow rate was set at 0.5 mL/min and the sample injection volume was 5 μL.

Conditions for mass spectrometry

A Triple TOF 5600 Mass spectrometer (AB SCIEX, Framingham, USA) equipped with ESI source was operated both in negative and positive ion modes. The operating mass parameters were set as follows: scan range, 50-1500 Da; ion spray voltage, 4.5 kV(ESI-) and 5.5 kV (ESI+); ion source heater, 600°C; nebulizing gas, 60 psi; drying gas, 60 psi;

curtain gas, 40 psi; collision energy, 40 eV; declustering potential, 100 eV; with a collision energy spread of ±15 V.

Data processing and statistical analysis

Components identification

The identification of WDD components were analyzed by PeakView Software. Firstly, all reported chemical components of six herbs and WDD were collected by different databases, including PubMed, SciFinder and CNKI. In the next step, the structures of compounds were searched in online mass databases (e.g., http://www.chemspider.com/, https://pubchem.ncbi.nlm.nih.gov/). Then, the compounds were preliminarily or explicitly inferred by comparing the structures, retention time and ion fragments with reference standards, relevant literature, software and databases (e.g., http://www.hmdb.ca/, http://www.massbank.jp/). Finally, the compound library was established, which including information on the name, molecular weight, molecular formula, chemical structure and related reference.

Comparation between different processes

The chromatographic peaks of samples with different processes were extracted, filtered and identified by software Makerview1.2.1. The parameters were set as follows: extraction range of retention time, 1-33 min; precursor ions, 50-1500; allowed deviation of retention time, 10 ppm; noise threshold, 8000. The obtained data was imported into SMICA-P 13.0 software to obtain PLS-DA score, OPLS-DA score, S-plot figure and

VIP values. Then, P value of t-Test and fold change (FC) value could be obtained by Makerview1.2.1 software. Finally, the differential components were imported into the website of MetaboAnalyst (https://www.metaboanalyst.ca/) to form cluster heat map, and other mapping was finished by GraphPad 7 software [17-18].

Results

Chemical profiling of WDD by UPLC/Q-TOF-MS/MS

A total of 121 components of WDD in the positive and negative ion mode were identified, including 23 organic acids, 69 flavonoids, 4 amino acids, 5 phenylpropanoids, 6 triterpenoid saponins and 14 other components. In comparison with each single decoction, 26 components of WDD were found from Banxia, 29 from Zhuru, 78 from Zhishi, 53 from Chenpi, 26 from Shengjiang, 61 from Gancao, and 20 common components from six herbs. The total ion chromatography was shown in Figure 1 and the identified components were summarized in Table 1.

Identification of Organic acids

Aromatic ring replaced by phenolic hydroxyl, acrylic acid and fatty acid were the basic structures of organic acids in WDD. They were easy to lose H_2O, COOH group and break at carbonyl to form fragment in negative ion mode. Compound 15 (t_R=2.494) was identified to give an example. It produced precursor ion 353.0877 $[M-H]^-$ and MS2 fragments at m/z 191.0570 $[M-H-C_9H_6O_3]^-$, 179.0341 $[M-H-C_7H_{10}O_5]^-$, 135.0459 $[M-H-C_8H_{10}O_7]^-$. Furthermore, the precursor ion $[M-H]^-$ of compound 5 (t_R=0.670) and

compound 17 (t_R=4.313) were at m/z 191.0554 and m/z 179.0340, suggesting that they might be the cleavage products of compound 15. After comparing with the retention time and fragments of the reference substances, compound 15 was determined as chlorogenic acid. Compound 5 lost a H_2O, CH_4O_3 or $C_3H_6O_4$ group to form fragment at m/z 173.0440 $[M-H-H_2O]^-$, 127.0394 $[M-H-CH_4O_3]^-$ or 85.0297 $[M-H-C_3H_6O_4]^-$, respectively. Compound 17 formed fragment at m/z 135.0453 $[M-H-CO_2]^-$ by losing CO_2 at the end of the acrylic acid, which was consistent with the reference [19]. Therefore, compound 5 and 17 were speculated as quinic acid and caffeic acid respectively. The cleavage regularity was shown in Additional file 2-1.

Identification of Flavonoids

Flavonoids included flavone, isoflavone, flavanone, chalcone, etc. In this study, liquiritin, naringenin-7-O-rutinoside, naringin, hesperidin and neohesperidin were identified to be the main flavonoid components of WDD. Taking compound 65 (t_R=13.237) as an example for identification, it produced precursor ion 579.1687 $[M-H]^-$ and formed fragments at m/z 459.1118 and 119.0504 through RDA cleavage reaction, or formed genin fragment at m/z 271.0581 through breaking the oxyglycoside bond. The genin fragment produced 151.0028 and 119.0504 through RDA cleavage reaction, then the ions of m/z 151.0028 was rearranged to formed m/z 107.0136. Compared with the reference substances and relevant literature [20, 21], compound 65 was ultimately confirmed to be naringin and compound 90 was naringenin. After losing the C7 glucose, compound 63 (t_R=12.910) was found to contain fragment information

of naringenin at m/z 271, therefore it was identified as prunin [21, 22]. The cleavage regularity was shown in Additional file 2-2.

The structure with terminal carbon atoms of glycosyl and the flavonoid carbon atoms connected directly was named as flavone with C-glycosides, such as schaftoside and isoschaftoside. In the present study, the precursor ion of schaftoside was 563.1401 $[M-H]^-$. It formed a series of fragments at m/z 503.0085 $[M-H-60]^-$, 473.1025 $[M-H-90]^-$, 443.0896 $[M-H-120]^-$, 383.07495 $[M-H-180]^-$, 353.0635 $[M-H-210]^-$, which were considered as the characteristic fragment of C-glycosides ring cleavage reaction [15]. In Additional file 2-3, there were four ring cleavage regularity: A was glucose 0, 3 bond cleavage with $C_3H_6O_3$ (m/z 90) removed; B was glucose 0,2 bond cleavage with $C_4H_8O_4$ (m/z 120) removed; C was arabinose 0,3 bond cleavage with $C_2H_4O_2$ (m/z 60) removed; D was arabinose 0,2 bond cleavage with $C_3H_6O_3$ (m/z 90) removed. It was shown that fragment at m/z 503.0085 was formed from C cleavage; m/z 473.1025 could be formed after A or D cleavage; B cleavage could form fragment at m/z 443.0896; m/z 383.07495 could be formed when B, C or A, D cleavage simultaneously; m/z 353.0635 could be formed when B and D cleavage simultaneously. Schaftoside and isoschaftoside were isomers, the bonding position of hexose and pentose was the clear difference between them. It was finally identified compounds 32 (t_R=8.461) and 40 (t_R=9.476) to be schaftoside and isoschaftoside respectively by relevant literature [23, 24].

Identification of Phenylpropanoids

Multiple oxygen atoms, hydroxyl and methoxy groups connected with aromatic rings were the structure features of phenylpropanoid molecules, and a series of fragment could be seen in MS2 after the loss of CO, H_2O, methyl and methoxyl continuously. In this study, 7-hydroxycoumarin, daphnetin, esculin and other phenylpropanoid components were identified in WDD. Taking esculin (t_R=4.263) as an example. The precursor ion was 339.0697 $[M-H]^-$, which formed the fragment at m/z 177.0191 $[M-H-C_6H_{10}O_5]^-$ and 133.0305 $[M-H-C_6H_{10}O_5-CO_2]^-$. The cleavage regularity was shown in Additional file 2-4. Therefore, it was assumed that the compound was esculin by reviewing the related literature [25, 26].

Identification of Triterpene saponins

Triterpene saponins were effective components from Radix Glycyrrhizae in WDD. In this study, six triterpenoid saponins were identified, and all of them were glycyrrhizic acid derivatives. Taking compound 104 (t_R=28.172) as an example, its precursor ion was 339.0716 $[M-H]^-$. The fragments at m/z 645.3635 [M-H-Glucuronide acid]$^-$, 469.3273 [M-H-2 Glucuronide acid]$^-$, 351.0555 [M-H-Glycyrrhetinic acid]$^-$, 193.0353 [M-H-Glycyrrhetinic acid-Glucuronide acid]$^-$ were produced after MS2 cleavage. The cleavage regularity was shown in Additional file 2-5. Combined with related literatures [27-29], fragment at m/z 469.3273 was identified as glycyrrhetinic acid and the compound 104 was glycyrrhizin.

Variation analysis of different processes based on chemometrics

Analysis of PLS-DA model

In the permutation test (Figure 2B), the sequential order of the categorical variable Y was randomly changed many times (n=200) and a corresponding PLS-DA model (Figure 2A) was established on each occasion. After that, the R2 and Q2 values of the stochastic model were obtained, which were directly proportional to the explanatory power and predictive power of the model respectively [30, 31]. In this model, the intercept values of R2 and Q2 were 0.780 and -0.571 respectively and all of them on the left were lower than those on the right, meanwhile the intercept of the regression line of Q2 was negative. The results suggested that this method was of great importance in preventing the test model from overfitting, as well as for evaluating the statistical significance of the model.

As shown in Figure 1A, each point represented a sample. All the points excepted one outlier fell within the 95% confidence interval, and the green, blue, red point represented ADP, MDP, MAPP group respectively. Apparently, each group could be separated clearly, indicating that there were some significant differences among these three processes. Compared with the ADP group, the sample distribution of the MDP group was more dispersed. It might be related to the improvement of extraction efficiency that played the effect of magnifying the intragroup differences. In comparison with the MDP group, the intragroup difference in the MAPP group was smaller, possibly caused by the alcohol precipitation. In addition, the three processes were arranged from right to left, which reasonably reflected the progressive relationship that "MAPP came from MDP and MDP came from ADP" to a certain extent.

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287 ***Variation analysis between ADP and MDP***

288 In order to identify the potential components that contributed significantly to the
289 differences between two groups, OPLS-DA model with supervised analysis was used
290 in this study. It could enhance the classification effect of samples, as well as the
291 effectiveness and analytical ability of the model based on the PLS-DA model [32]. The
292 verification result of ADP-MDP OPLS-DA model was depicted in Figure 3A, which
293 showed better separation effect and stronger explanatory power than PLS-DA model.
294 Permutation test (R2X=0.396, R2Y =0.971, Q2=0.865, $P < 0.05$) indicated the model
295 was credible.

296 In the S-plot (Figure 3B), all points were roughly distributed in an "s-type" pattern.
297 Each point in the figure represented a component, and the component closer to both
298 ends of the "s-type" pattern showed greater contribution to the difference of the
299 processes [33]. Variable importance on projection (VIP) was mainly used for screening
300 variables in PLS model. The VIP value represented the importance of variables to
301 model fitting [34]. In Figure 3C, each column represented a component. The higher the
302 column was, the larger the VIP value was, and the more significant the component was
303 to the differences of two processes. It could be seen that there were 51 components
304 whose VIP value were greater than 1. T-Test could be conducted on Makerview1.2.1
305 software to obtain the *P* value, and the difference was considered statistically significant
306 when $P < 0.05$. It was found that 81 components were significantly different with $P <$
307 0.05 between ADP and MDP. Fold change (FC) referred to the difference multiple of

sample variable expression. It was one of the difference analysis methods to describe the degree of change from an initial value to a final value. In this study, the components whose FC value were higher than 1.5 or lower than 0.67 were considered to contribute more to the differences, and there are 174 compounds that met this condition. Besides, if $log_2FC < 0$, the content of each component in MDP was up-regulated, indicating the converse expression if $log_2FC > 0$. In conclusion, 51, 81 and 174 components met the conditions of VIP > 1, P <0. 05 and FC > 1.5 (or < 0.67) respectively, and 24 of which met the above 3 conditions at the same time. The result was shown in Venn diagram (Figure 3D).

The 24 differential components selected from ADP - MDP were analyzed to obtain the cluster heat map (Figure 3E). Each column in the figure represented a sample, and every pixel indicated a component. The color of each pixel varied from deep blue to deep red showed the degree of expression which varied from low expression to high expression [33]. According to the result of clustering analysis, samples of every group could be better clustered except the B2 sample of MDP, indicating the significant difference between ADP and MDP could be related to the 24 differential components. Moreover, it showed that some of the components were grouped together, which suggested these components might be different ion forms of the same compound or belong to the same class of compounds.

In the 24 differential components, 13 components could be identified as sucrose, salicylic glucuronide, benzoic acid, naringenin-7-O-rutinoside, hesperidin, neodiosmin, neohesperidin, liquiritigenin, fortunellin, poncirin, naringenin, hesperetin and 6-

gingerol, respectively, and other compositions remained to be discerned. The detailed information was shown in Additional file 3-1. The chromatographic peak areas of 13 components in ADP and MDP were preliminarily compared in Figure 4.

Variation analysis between MDP and MAPP

By processing data in the same way, permutation test showed the OPLS-DA model of MDP-MAPP was unoverfitted (R2X=0.504, R2Y=0.931, Q2=0.775, $P < 0.05$). Score graph, S-plot graph and VIP graph were shown in Figure 5 (A, B, C). In Figure 5D, a total of 18 components met the requirements of VIP $>$ 1, $P < 0.05$ and FC $>$ 1.5 (or < 0.67) at the same time. The cluster heat map of 18 compounds (Figure 5E) showed samples could be better clustered in MDP and MAPP. In the 18 differential components, only 8 compounds could be identified as citric acid, sucrose, pentonic acid, gluconic acid, quinic acid, mucic acid, salicylic glucuronide and neohesperidin, respectively. The detailed information was shown in Additional file 3-2 and the preliminarily comparation of 8 components in MDP and MAPP was shown in Figure 6.

Discussion

Selection of LC and MS conditions

Different mobile phases were compared in the experiment. It was found that the peaks of WDD were separated better with acetonitrile-0.1% formic acid solution. In general, components with acidic groups were suitable for analysis in the negative ion mode, while the positive ion mode was suitable for basic groups [35, 36]. The main

components of WDD were flavonoids with multiple hydroxyl or organic acids with carboxyl, all of them were easy to form stable oxygen anion in negative ion mode. Therefore, the negative mode was used mainly while the positive ion mode was used as auxiliary to improve the accuracy and credibility of the experimental results.

Analysis of the identified components of WDD

The components of WDD were effectively identified by matching the chemical structures, retention time and ion fragments with reference standards, relevant literature and online mass databases. Twenty-eight components were verified by comparing with reference standards. Other isomers of WDD remained to be further investigated by nuclear magnetic resonance technology. The results showed that flavonoids were the main components of WDD and mainly from Zhishi, Chenpi and Gancao. It had been reported that flavonoids showed significant anti-oxidant, anti-inflammatory, anti-bacterial and neuro-protective activities [37-42], which were consistent with the main pharmacological effects of WDD. So, flavonoids could be speculated to be the main active components of WDD. It would provide ideas for the quality control of WDD. However, further pharmacodynamic studies were needed to verify it, which might be the next task of our group.

Variation analysis between different processes

The results revealed that the relative content of compounds in MDP was higher than that in ADP, suggesting the extraction efficiency of modern technology was greater than

that of ancient process. However, this conclusion remained to be confirmed by the next study of absolute quantitative. It could be speculated that if the ancient dosage was followed in the study of CP, the efficacy of modern formulae would be stronger than that of ancient prescription, or side effects would even occur. So, it was suggested that the content of active ingredients should be strictly controlled during the research of CP, as well as the oral dosage of modern formulae would be determined according to actual condition.

WDD samples of ADP and MDP were better clustered, which could be related to the 24 differential components between these two processes. Among the 13 identified compounds, naringenin-7-O-rutinoside, hesperidin, neohesperidin, naringenin, hesperetin were the main components of Zhishi and Chenpi, as well as liquiritigenin and 6-gingerol were from Gancao and Shengjiang respectively. Most of the differential components were from Zhishi and Chenpi, indicating that the differences between ADP and MDP could be significantly generated by these two herbs. Furthermore, some differential components had been reported to be connected with the neurological diseases. For example, benzoic acid could affect the therapeutic effect of Alzheimer's disease to a certain extent [43]; Hesperidin had certain effect on anti-inflammatory [44], neuroprotective [45] and the improvement of depression [46]; Neohesperidin contributed to the treatment of depression and Alzheimer's disease [47, 48], as well as glycyrrhizin and naringin contributed to antidepressant and neuroprotective [49]; 6-gingerol was able to inhibit oxidative stress and inflammatory response [50], etc. Therefore, it was speculated that different preparation processes of WDD would

produce different clinical efficacy under the influence of differential components.

In addition, the process of alcohol precipitation could affect 13 components prominently in WDD, and 8 of which could be identified. There was a downward trend on the content of citric acid, mucic acid, salicylic glucuronide and neohesperidin after alcohol precipitation. It could be explained that small molecules in the solution were wrapped or loaded on the precipitation when starch, polysaccharides, proteins and inorganic salts were removed [51]. However, there was an opposite trend on content of sucrose, pentonic acid, gluconic acid and quinic acid, which might be related to the cracking of some components during alcohol precipitation [52]. In modern preparation research, alcohol precipitation technology was used to purify decoction, so as to facilitate preparation and reduce dosage. As polysaccharide also showed the pharmacological activity [53], it had yet to be explored whether alcohol precipitation process could affect the efficacy of WDD, and the rationality and necessity of alcohol precipitation should also be considered.

Conclusions

In this study, UPLC/Q-TOF-MS/MS in both positive and negative ion mode was used to analyze the components of WDD. Ultimately, 121 components were identified, including 23 organic acids, 69 flavonoids, 4 amino acids, 5 phenylpropanoids, 6 triterpenoid saponins and 14 other compounds. It was found that 29%, 22%, 19%, 11%, 10% and 9% identified components were from Zhishi, Gancao, Chenpi, Zhuru, Shengjiang and Banxia respectively. Since the pharmacological activities of flavonoids

had been proved, it was considered that flavonoids could be the main active ingredient of WDD. Additionally, Chemometrics was innovatively adopted to testify the significant differences between ADP, MDP and MAPP. The 24 components were considered to be related to the differences between ancient and modern technology, and 18 compounds could be affected in the process of alcohol precipitation. Most of the differential compositions were reported to have the same efficacy with WDD, which could provide bases for the study of active ingredient and quality control of WDD, as well as promote the development of WDD traditional classic preparation into modern preparation.

Supplementary information

Additional file 1: The regions and batch numbers of six herbs in WDD

Additional file 2: The MS2 spectra and cleavage regularity of compounds in WDD

Additional file 3: Detailed information on the differential components

Abbreviations

WDD: Wen-Dan decoction; TCM: traditional Chinese medicine; CP: classic preparation; UPLC/Q-TOF-MS/MS: ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; PLS-DA: partial least squared discriminant analysis; OPLS-DA: orthogonal partial least squared discriminant analysis; ADP: ancient decoction process; MDP: modern decoction process; MAPP: modern alcohol precipitation process; VIP: variable importance on projection; FC: fold change.

Acknowledgments

Thanks to Jinghua Pharmaceutical Group Co., Ltd for supplying the herb materials of WDD and thanks to Prof. Jianwei Chen for the identification.

Authors' contributions

LD and YC conceived and designed the experiments; XG performed the experiments and wrote the paper; ZL and PQ processed and analyzed the data; XZ provided assistance for the components identification; WZ gave great assistance for the fulfilment of this study.

Funding

This work was supported by Standardization Project of the National Administration of Traditional Chinese Medicine (ZYBZH-C-JS-30, China); Graduate Research and Innovation Project of Jiangsu Province (KYCX19_1265, KYCX19_1313); Natural Science Research Project of Colleges and Universities in Jiangsu Province (19KJB360004); Natural Science Foundation of Jiangsu Province (BK20190800).

Availability of data and materials

All data and materials are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

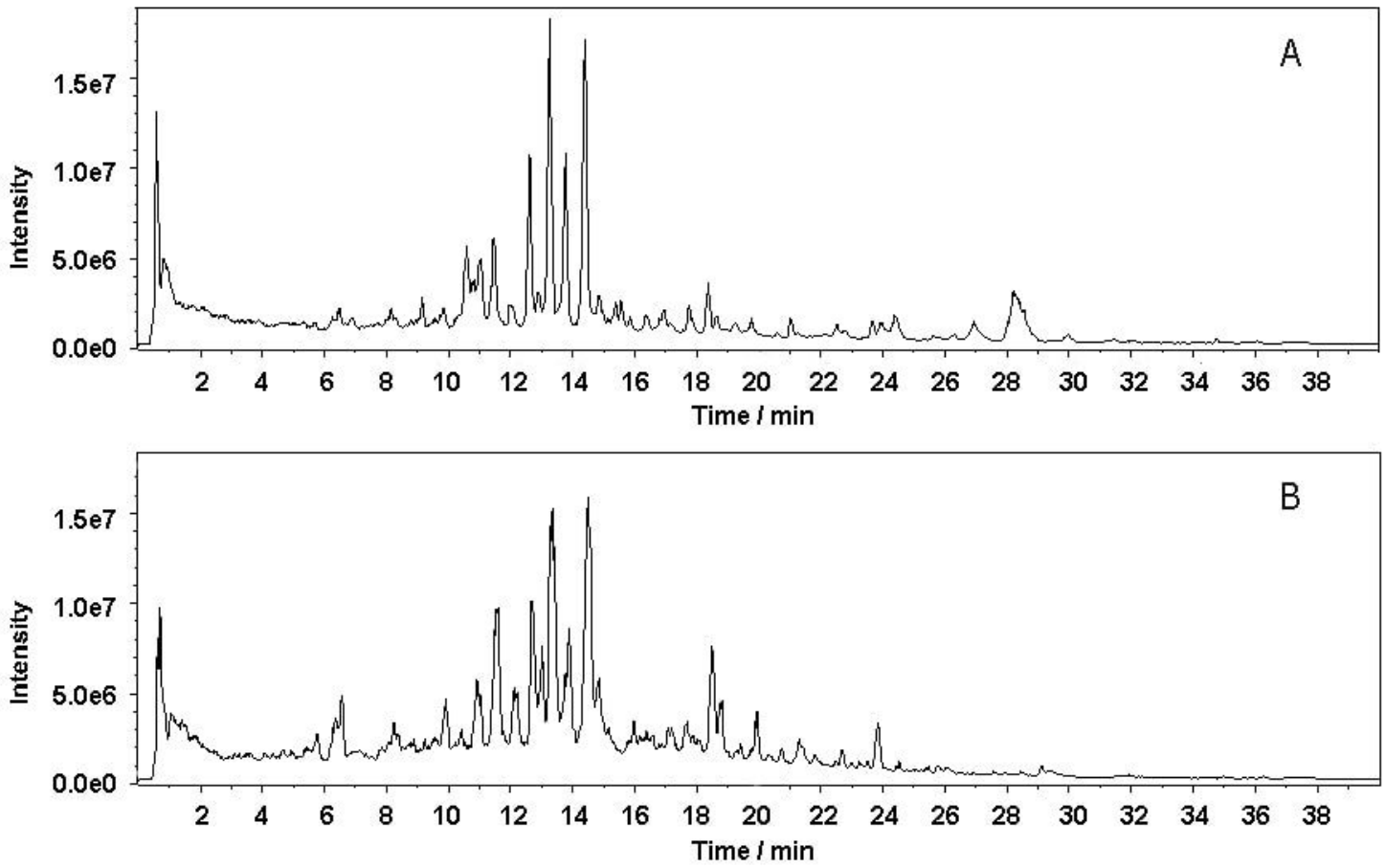

Figure 1

Total ion chromatography

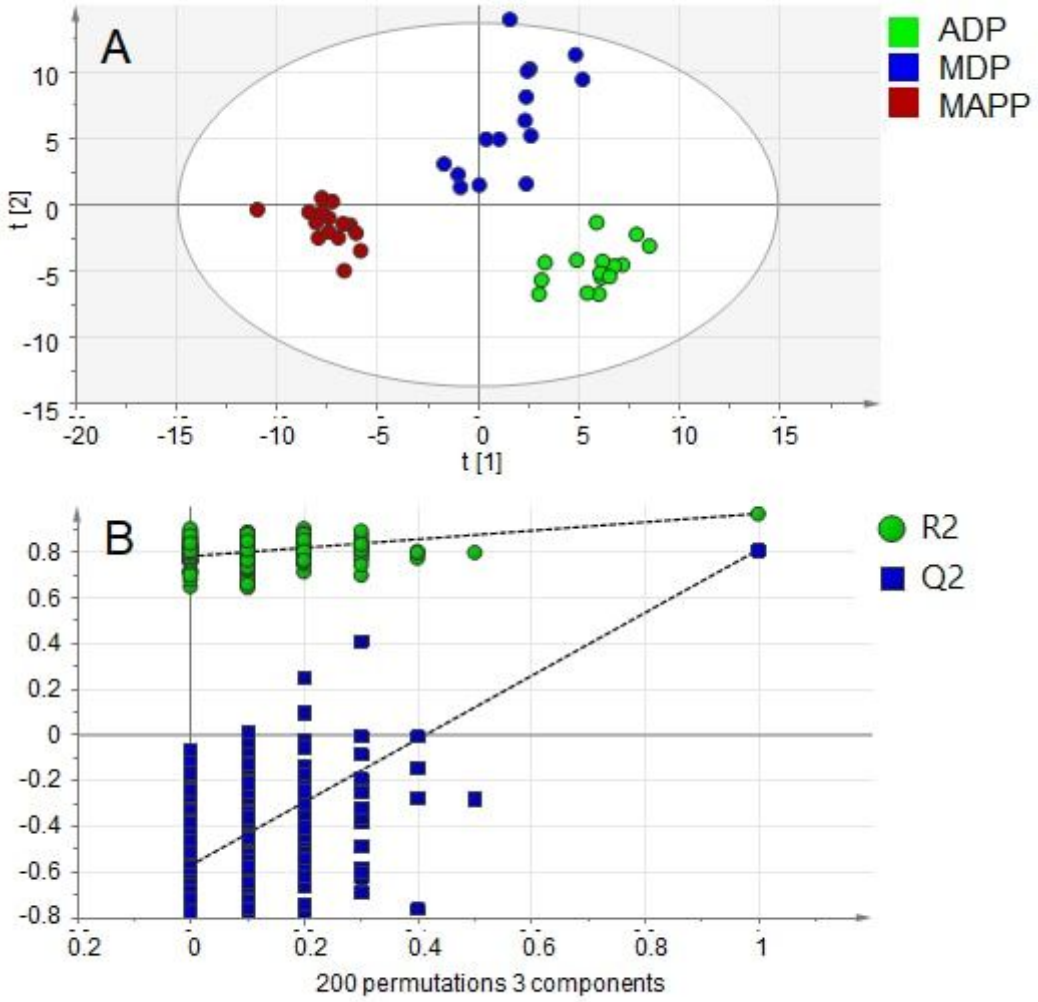

Figure 2

In the permutation test (Figure 2B), the sequential order of the categorical variable Y was randomly changed many times (n=200) and a corresponding PLS-DA model (Figure 2A) was established on each occasion.

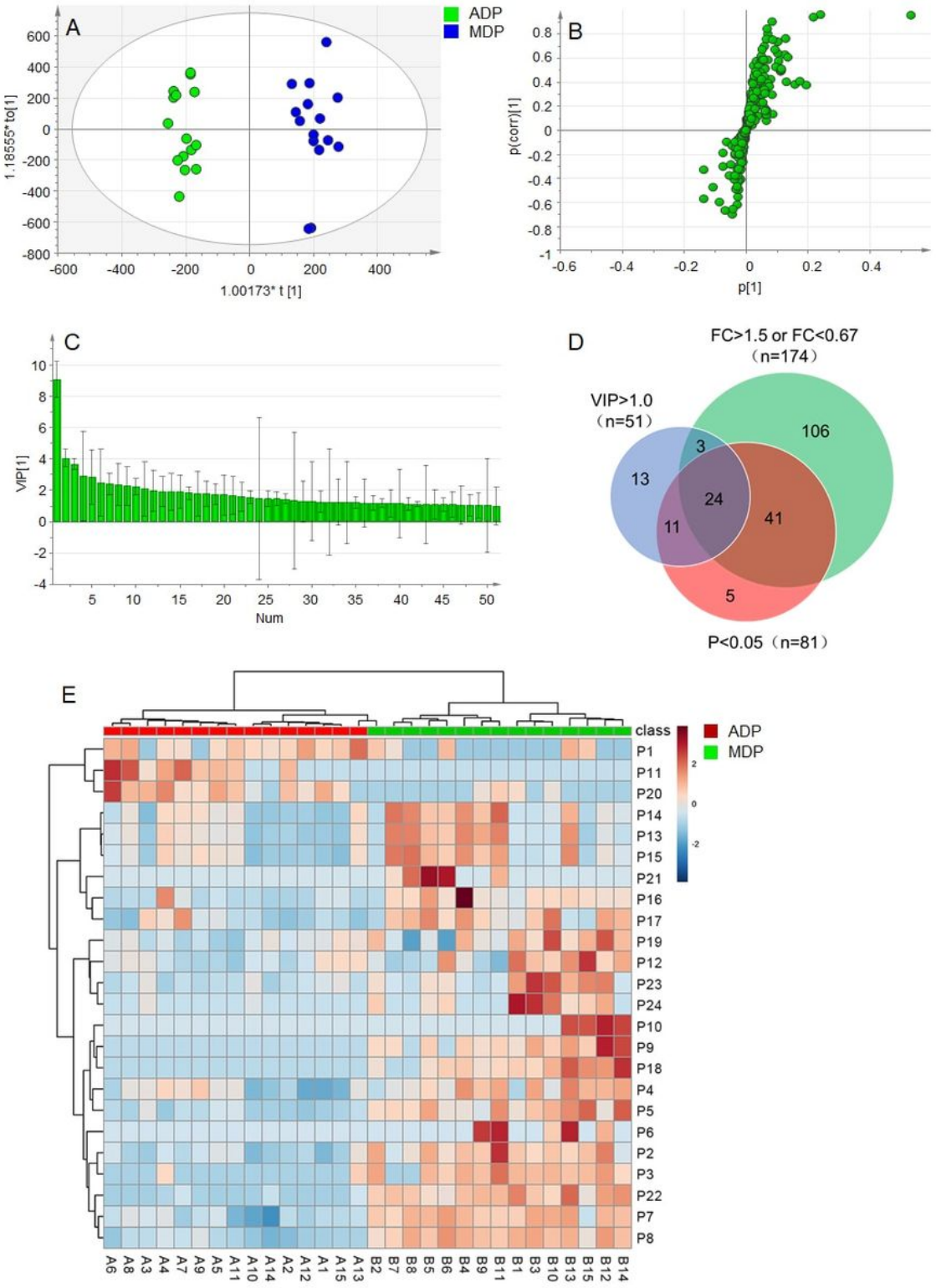

Figure 3

The verification result of ADP-MDP OPLS-DA model was depicted in Figure 3A, which showed better separation effect and stronger explanatory power than PLS-DA model. Permutation test (R2X=0.396, R2Y =0.971, Q2=0.865, P < 0.05) indicated the model was credible. In the S-plot (Figure 3B), all points were roughly distributed in an "s-type" pattern. Each point in the figure represented a component, and the component closer to both ends of the "s-type" pattern showed greater contribution to the difference of the

processes [33]. Variable importance on projection (VIP) was mainly used for screening variables in PLS model. The VIP value represented the importance of variables to model fitting [34]. In Figure 3C, each column represented a component. The higher the column was, the larger the VIP value was, and the more significant the component was to the differences of two processes.

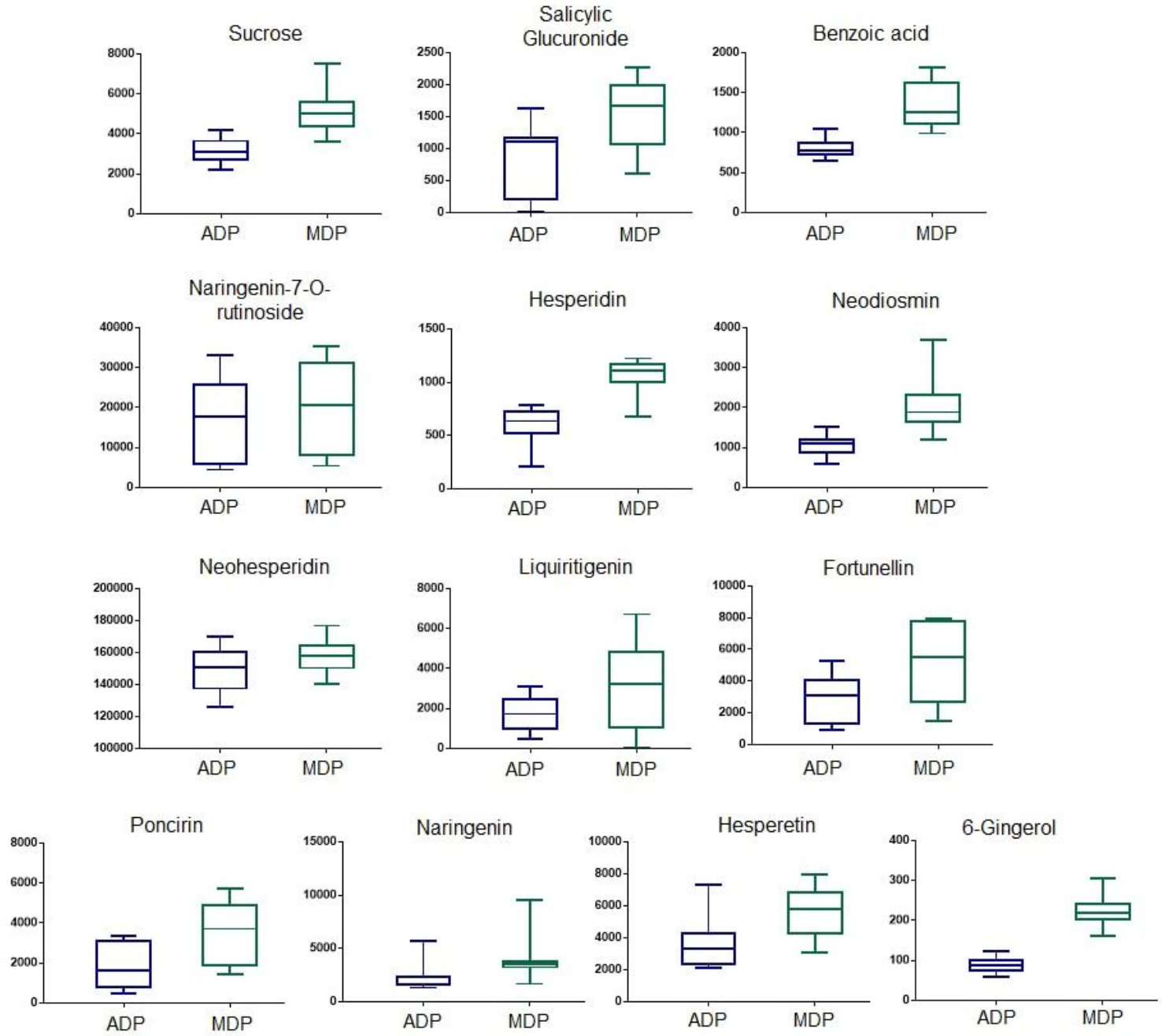

Figure 4

The chromatographic peak areas of 13 components in ADP and MDP were preliminarily compared

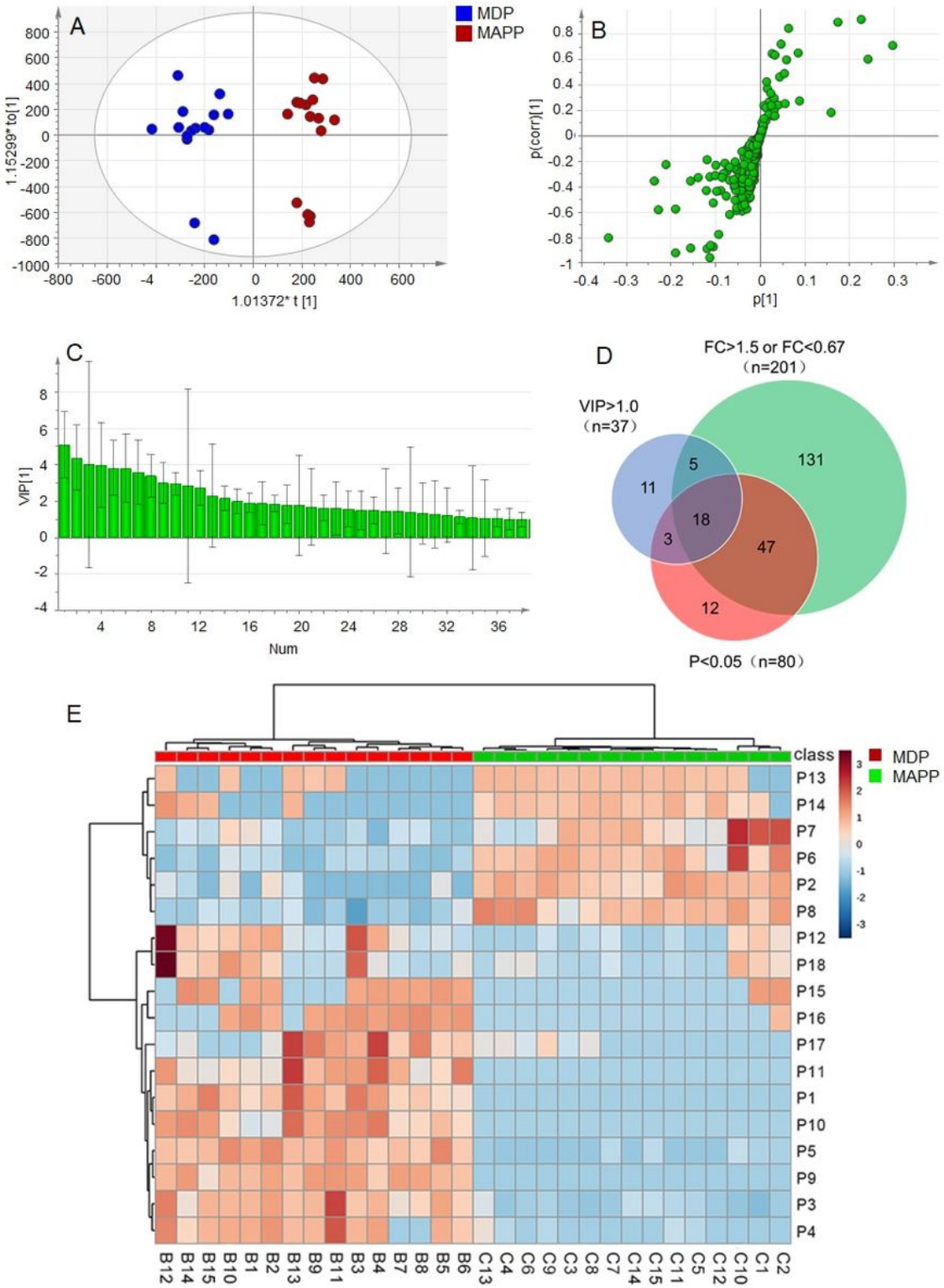

Figure 5

Score graph, S-plot graph and VIP graph were shown in Figure 5 (A, B, C). In Figure 5D, a total of 18 components met the requirements of VIP ≥ 1, P ≤ 0.05 and FC ≥ 1.5 (or < 0.67) at the same time. The cluster heat map of 18 compounds (Figure 5E) showed samples could be better clustered in MDP and MAPP.

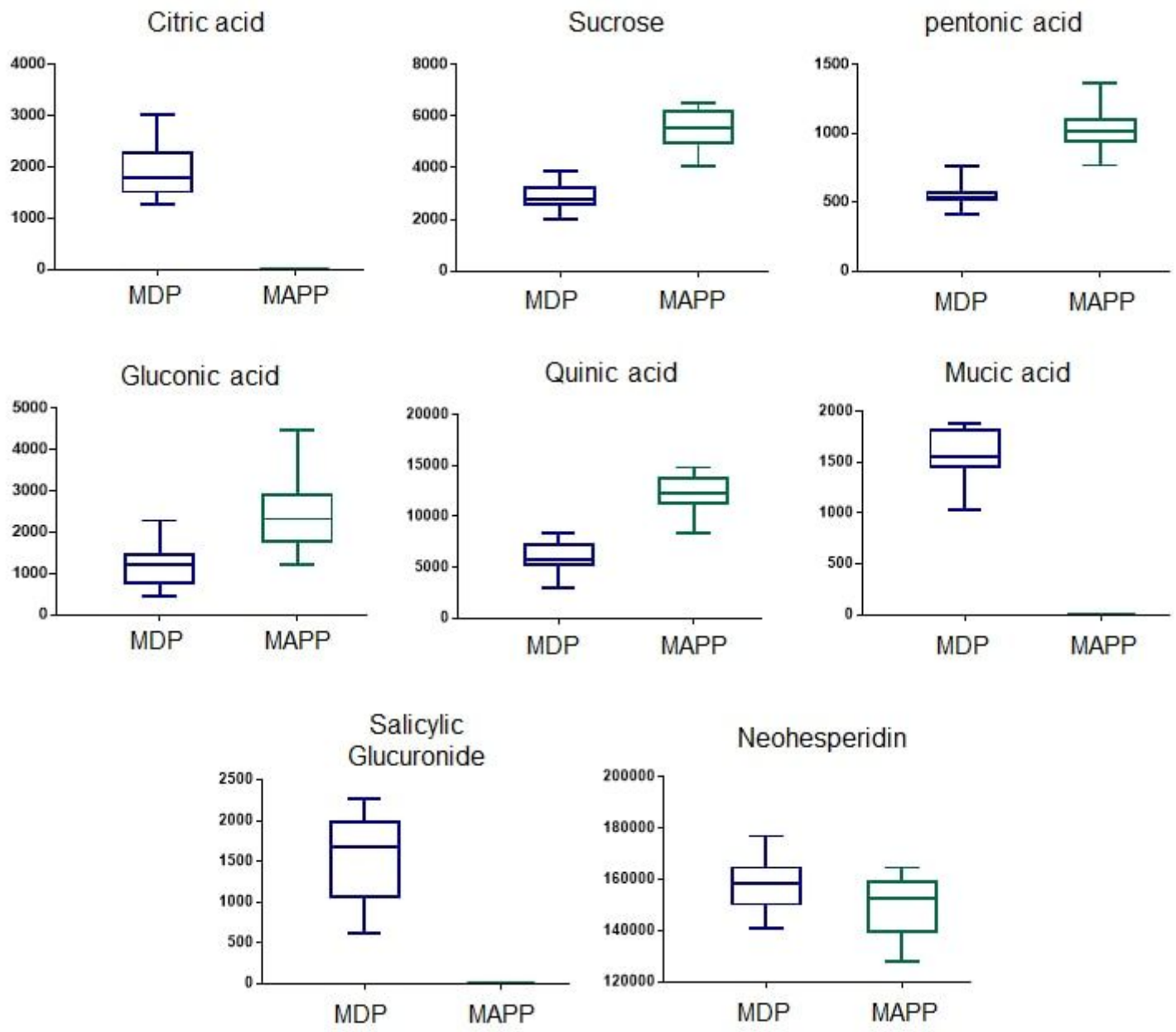

Figure 6

the preliminarily comparation of 8 components in MDP and MAPP

Supplementary Files

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