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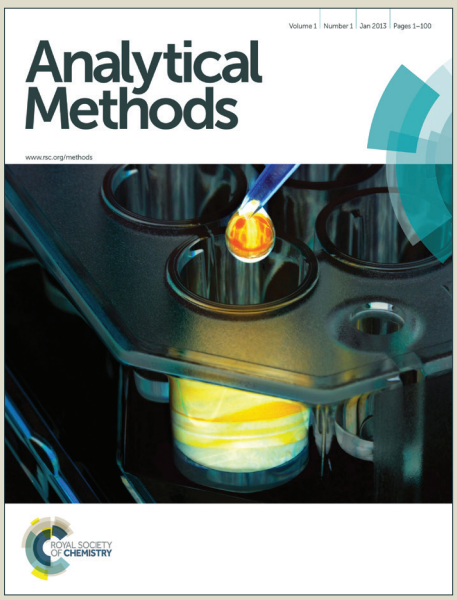

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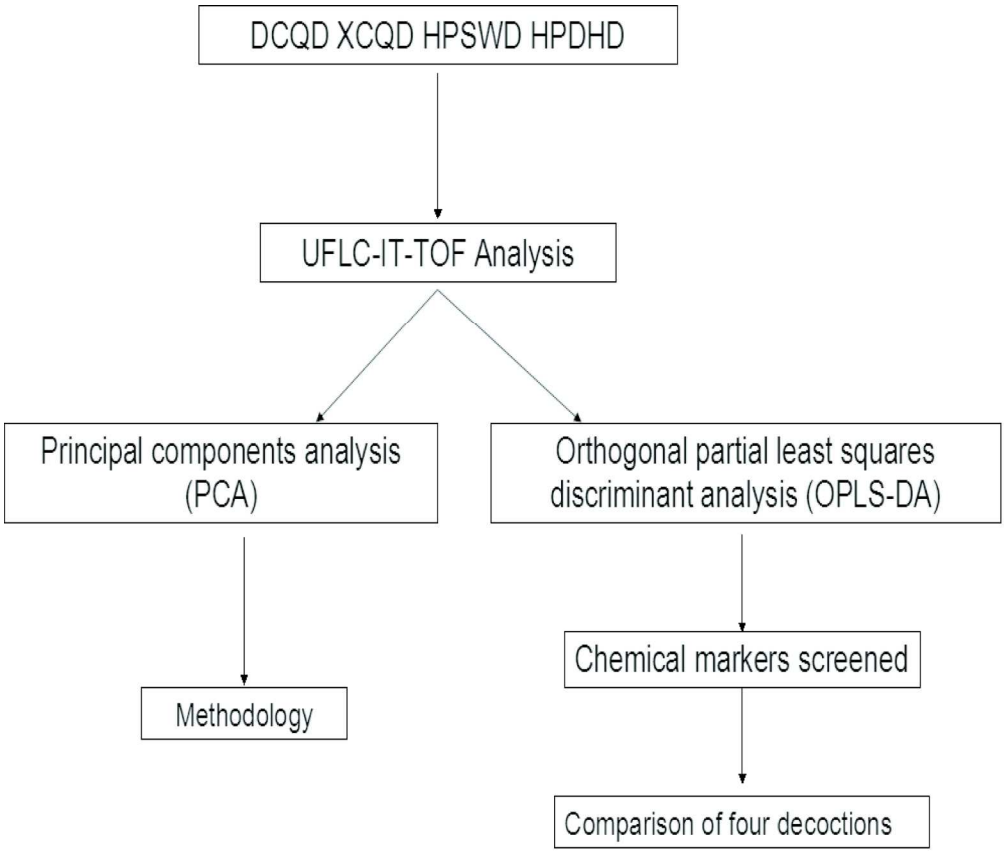

graphical abstract
182x154mm (300 x 300 DPI)

Chemical Differentiation of Da-Cheng-Qi Decoction and Its Three Analogous Decoctions Using UFLC-IT-TOF/MS-based Chemomic and Chemometric Approach

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Abstract

A rapid, sensitive and efficient ultrafast liquid chromatography (UFLC)-ion trap time-of-flight mass spectrometry (IT-TOF/MS) coupled with multivariate statistical analysis method has been developed and validated for evaluating chemical differentiation of Da-Cheng-Qi Decoction (DCQD) and its three analogous decoctions. DCQD, Xiao-Cheng-Qi Decoction (XCQD), Hou-Pu-San-Wu Decoction (HPSWD) and Hou-Pu-Da-Huang Decoction (HPDHD), four similar prescriptions of traditional Chinese medicines (TCMs) widely used in the treatment of gastrointestinal diseases, were decocted according to literatures and then subjected to UFLC-MS analysis. The data matrix integrating of retention time (t_R), mass-to-charge ratio (*m/z*) pairs, ion intensities and sample codes were processed with principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The results indicate significant differences existing among these four decoctions and twenty-six chemical markers were screened out, of which five were from the root and bark of *Radix et RhizomaRhei*, eight from the bark of *Magnolia officinalis Rehd* and the rest from *Fructus Aurantii Immaturus* after identification by retention time and MS data. The underlying relationship between the difference in quantity of chemical markers and the difference in pharmacological effect of four similar decoctions was discussed. The LC/MS method combined with chemomic and chemometric approach provided a global research direction of chemical differentiation of DCQD and its three analogous decoctions and laid a foundation for the subsequent comparative pharmacological study of the four decoctions *in vivo*.

1. Introduction

DCQD, XCQD, HPSWD and HPDHD are well-known and popular TCMs in China and other Asian countries for the treatment of gastrointestinal diseases. They were described in Treatise on Febrile Diseases (Shang-Han-Lun in Chinese), a very famous TCM clinical work written by Zhang Zhongjing in about A.D. 200-205. The four multi-herb decoctions share three common constitutions, which are the root and bark of *Radix et Rhizoma Rhei*, the bark of *Magnolia officinalis Rehd* and *Fructus Aurantii Immaturus*. Besides, DCQD has additional one: *Mirabilitum* (crystals of sodium sulfate), a mineral drug that is considered as a carrier in DCQD [1].

The pharmaceutical effects and clinical applications of four multi-herb decoctions are diverse on account of different proportions of herbs and disparate ways of decoction. DCQD is effective in diseases like acute intestinal obstruction, acute cholecystitis and acute appendicitis. Researchers have shown that DCQD was also helpful in treating post-traumatic respiratory distress syndrome, drug-induced intestinal obstruction and organophosphorus pesticide intoxication [2-5]. XCQD is used for treating patients with bloating, constipation, moist fever and a sinking pulse. XCQD is also effective in diseases like adhesive intestinal obstruction, enteroplegia, chronic gastritis [6-8]. The purgative effect of DCQD is much stronger than XCQD. According to Shang-Han-Lun, HPDHD is used for cough wheeze, wet pleurisy and slippery pulse. HPDHD could be used for treating patients with acute gastric dilatation. HPSWD is mainly focused on diseases such as chest congestion, abdominal distension and HPSWD is also effective in treating adynamic ileus and constipation [9-10].

It was well known that TCMs played a role through a multi-components and multi-targets manner. The multiple constituents were usually responsible for its therapeutic effects by synergistic and/or antagonistic interaction. The analysis of multiple active constituents of TCMs might be helpful for connecting chemical components and clinical effects, and furthermore facilitate the curative mechanism investigation of them. The different proportions of herbs and disparate ways of decoction in these four multi-herb decoctions result in various pharmaceutical effects and clinical applications. Several published papers have reported the simultaneous quantification of active constituents of DCQD in animal plasma [11-17], the comparative study of anthraquinones in XCQD, HPSWD, HPDHD [18]. However, all these above only arbitrarily focused on a limited number of TCM components and thus did not reveal the multi-component characteristics of TCM prescriptions. Chemomic research is concentrated on discovering and confirming effective chemical substances and critical medicinal compositions gradually from the whole components in TCMs by a holistic approach [19-20]. Up to now, the chemical comparison of

four decoctions based on chemomic from the overall TCMs has never been reported. So, a rapid, sensitive and efficient UFLC-IT-TOF/MS combined with chemomic and chemometric approach was firstly developed to detect the chemical differentiation among these four decoctions which could be extended to the analysis of other analogous prescriptions of TCMs.

2. Experimental

2.1 Chemical and reagent

Reference standards of magnolol, rhein, synephrine, catechin, hesperidin and naringin were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Neohesperidin was purchased from Shanghai R&D Center for Standardization of Chinese Medicines (Shanghai, China).

Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Formic acid (analytical grade) and dimethyl sulfoxide (DMSO) (analytical grade) were obtained from Nanjing Chemical Reagent (Nanjing, China). Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

The herbs used in the experiment including the root and bark of *Radix et Rhizoma Rhei*, the bark of *Magnolia officinalis Rehd*, *Fructus Aurantii Immaturus* and *Mirabilitum* were purchased from a traditional Chinese medicinal store in Nanjing, China and authenticated by Assoc Prof Rui Song (Key Laboratory of Natural Medicine, China Pharmaceutical University, Nanjing, China).

2.2. Preparation of stock solutions of reference standards

The stock solutions of all the above-mentioned reference standards were prepared after the correcting for purity and were stored at 4 ℃. The stock solution of magnolol, rhein, synephrine, catechin, hesperidin, naringin and neohesperidin were prepared at 1 mg mL^{-1} in methanol and were further diluted with methanol to prepare the working solutions at 5.0 μg mL^{-1}. The stock solution of rhein and neohesperidin was diluted by methanol with a bit of DMSO added.

2.3. Preparation of DCQD, XCQD, HPSWD, HPDHD and three single-herb decoctions

The four multi-herb decoctions and three single-herb decoctions of the root and bark of *Radix et Rhizoma Rhei*, the bark of *Magnolia officinalis Rehd* and *Fructus Aurantii Immaturus* were prepared according to the method and procedure described in Shang-Han-Lun [18, 21-22] and were optimized which shown in supplementary materials. The proportions of herbs in single-herb and multi-herb decoctions were shown in Table 1.

2.4. Sample preparations

1 mL decoction was diluted to a 5 mL volumetric flask by distilled water with 1 mL DMSO added. After being vortexed, the solution was filtered through 0.22 μm nylon filter film, and then analyzed by LC-IT-TOF.

2.5. Instrumentation and operation conditions

Liquid chromatographic separation and mass spectrometric detection were achieved by employing UFLC coupled with IT-TOF/MS via electrospray ionization (ESI) interface (Shimadzu, Japan). The analytical column was ZORBAX SB-C18RHT(100 mm×2.1 mm I.D.,1.8 μm). The column and automatic sampler were maintained at 35 ℃ and 4 ℃, respectively and the injection volume was 3 μL. The gradient elution was 40 min at a flow rate of 0.3 mL min^{-1} with the mobile phase containing water with 0.1 % formic acid (mobile phase A) and methanol (mobile phase B). From 0.1 min to 20 min, mobile phase B was increased linearly from 10 % to 60 %. Then, mobile phase B was linearly increased to 100 % within 5 min and kept at 100 % for 5 min. At 31 min, the proportion of mobile phase B was adjusted to 10 % for equilibration for 10 min.

Mass spectra in both positive and negative ionization mode were obtained simultaneously in a full-scan operation with a scan range of 50-1000 *m/z* by switching the interface voltage between 4.5 kV and -3.5 kV in each 0.1 s. The flow rate of the nebulizing gas (N_2) was 1.5 L min^{-1} .The temperatures of the curved desorption line and the heat block were both 200 ℃, and the microchannel plate detector voltage was set to 1.60 kV. The pressure of the drying gas (N_2) was 100 kPa, and the ion accumulation time was set to 30 ms. Mass spectra and chromatograms were acquired and processed with LC/MS solution version 3.0 (Shimadzu, Japan).

2.6 Data preprocessing and analysis

The obtained data from LC/MS were processed by Profiling Solution version 1.1 (Shimadzu, Japan) for peak deconvolution and alignment. The method parameters were: width (5 s), slope (2,000 min^{-1}), retention time range: 0.5-30 min, ion *m/z* tolerance: 50 mDa, ion retention time tolerance: 0.5 min, ion intensity threshold: 10,000 counts. The selected retention time range discarded the injection time area where co-elution of polar constituents probably occurred. After completing the integration parameters, a report of peaks based on areas, retention time and *m/z* was generated for each sample. Signals of different samples were considered to be similar when they simultaneously fulfilled both retention time (0.5 min tolerance) and *m/z* value (50 mDa tolerance) criteria. The peaks from *Mangnolia officinalis* in four multi-herb decoctions were picked up by comparing with those in single-herb decoctions of *Mangnolia officinalis*. These screened data were processed according to the "80 % rule": only the

variables with values above zero in at least 80 % of either group were kept for the following analysis. Then, variables with relative standard deviation (RSD) lower than 30 % in quality control (QC) samples (prepared by combining equal aliquots from all decoction samples) were chosen, and the individual area was normalized against the sum of chosen signals (i.e. the common peaks for multi-herb decoction and single-herb of *Mangnolia officinalis*). Positive and negative ion data were independently preprocessed before merging. Then we performed Pareto transformation to stabilize the variance throughout the intensity range. The final data table was processed with PCA and OPLS-DA by SIMCA-P version 13.0 (Umetrics, Sweden). The corresponding variable importance (VIP value) was calculated in the OPLS-DA model. Then, the nonparametric Kruskal–Wallis rank sum test was performed to determine the significance of each marker, and the relevant false discovery rates based on the *p* value were estimated in the context of multiple testing. A chemical marker was selected when the VIP value was more than 1.0 and *p* value was less than 0.05. Similarly, we selected chemical markers from *Radix et Rhizoma Rhei* and *fructus Aurantii Immaturus* with the same method above.

2.7 Identification of potential chemical markers screened

Identification of chemical markers was achieved through a mass-based search followed by manual verification. The hypothetic identifications were verified by comparing the MS^2 fragmentation patterns, accurate molecular weights and retention time with those of authentic standard compounds or those data in the literature which had been published. The box-plot was used to visualize the variation in the levels of chemical markers in four multi-herb decoctions.

2.8 Method validation

In any LC-MS system, the sample unavoidably contacts directly with the instrument which results in changing response of chemical substances. Signal attenuation is not consistent in measurement. For this reason, it is necessary that QC samples are periodically analyzed throughout an whole analytical run. Data management can use the QC responses as the basis to evaluate the quality of the data, delete peaks with poor stability, correct the signal attenuation and concatenate batch data together after data acquisition and before statistical analysis. Within-batch run order is assigned stochastically to each sample so that the sample order is random but stratified.

Furthermore, for LC/MS analysis, several substances among chemical markers which had good intensity and peak shape in the chromatogram were selected to check repeatability, intra-batch and inter-batch precision during analytical batches. The repeatability of UFLC-IT-TOF-MS was evaluated as the relative standard deviations

(RSD, %) of peak areas in UFLC chromatograms using three replicates of the QC sample at different time intervals (0 h, 6 h and 12 h). The intra-batch precision was evaluated by determining RSD of peak areas of five replicates of the QC sample under the optimised condition in one day. For inter-batch precision, the measurement was conducted for three consecutive days. The chemical markers selected were as follows: catechin, rhein, magloside A, magnolol, naringin and neohesperidin.

3 Results and discussion

3.1 Method validation

Under the chromatographic and MS conditions, the major components in DCQD and its three analogous decoctions were well-separated and detected in 30 min. The representative base peak intensity chromatograms of DCQD were shown in Fig. 1.

Repeatability and precision of analytes were systematically studied. The results are presented in Table 2. Repeatability and precision (RSD) of analytes was found to be less than 8 % which demonstrated that the established method was robust and reliable.

3.2 Data preprocessing

After peaks had been picked with Profiling Solution, the peaks of *Radix et Rhizoma Rhei*, *Mangnolia officinalis* and *fructus Aurantii Immaturus* in four multi-herb decoctions were extracted by comparing with those in three single-herb decoctions respectively and screened with the "80% rule" and "RSD≤30% in the QC samples". Then the individual area was normalized against the sum of chosen signals (i.e. the common peaks for multi-herb decoction and single-herb) so as to remove the unwanted systematic bias in ion intensity within each sample in measurements. Futhermore, after extracting the single-herb peaks in four multi-herb decoctions respectively, "total area normalization method" can ignore the difference of the amount of single herbs in different decoctions in order to make different proportions of single herbs comparable. Multivariate statistical analysis was used to filter unmeaningful information from these large data sets. To reduce the importance of the variables with large intensities and avoid selection of the most abundant chemicals as significant, Pareto scaling was used, where each variable was divided by the square root of the standard deviation.

3.3 Statistical analysis

PCA, an unsupervised multivariate data analysis technique without using class information, was firstly performed to investigate whether five groups could be separated according to their differences in the chemical

compositions. All data were displayed as scores and loadings in a coordinate system of principal components resulting from data dimensionality reduction. As shown in Fig. 2A-2B, QC samples were clearly distinguished from five groups on the PCA scatter plot. The QC samples clustered tightly in both modes, showing the stability of the LC/MS platform throughout the whole run.

The PCA scatter plots of peaks from *Magnolia officinalis* were shown in Fig. 2A-2B. For both ESI+ and ESI- mode, samples of single-herb decoctions of *Magnolia officinalis* were separated from other groups which showed that the dissolution of *Mangnolia officinalis* was affected by other herbs added in four multi-herb decoctions. As PCA did not use the class information of the data, other informative characteristics are not obvious. Then, OPLS-DA, a supervised analysis technique which was performed to clarify the class partition, was employed to divide the different groups and screen chemical markers. The OPLS-DA plots (Figure 2C-2D) showed different groups were separated which declared that different amount of other herbs added leaded to different dissolution of *Mangnolia officinalis*. Due to similar amount of herbs in HPDHD and HPSWD, the two groups were very close in the OPLS-DA plot.

The model statistics, R^2X, R^2Y and Q^2 which are usually used for evaluation of OPLS-DA, showed improved model predictability and good ability to explain the variation between four multi-herb decoctions and single-herb decoctions. As a result, 19 (ESI+) and 26 (ESI-) chemical variables were screened out for further identification.

With the same method, we screened chemical markers of *Radix et Rhizoma Rhei* and *Fructus Aurantii Immaturus*. For *Radix et Rhizoma Rhei*, we obtained 16 (ESI+) and 24 (ESI-) chemical variables; and for *Fructus Aurantii Immaturus*, we got 34 (ESI+) and 48 (ESI-) chemical variables.The features left after each filtering step were shown in supplementary Table 1. The PCA and OPLS-DA plot of them were showen in Fig. A and Fig.B of supplementary materials.

3.4 Identification and verification of chemical markers

Twenty-six chemical markers are summarized in Table 3 with their corresponding retention time, molecular formula and measured mass. Seven of the markers were identified by authentic standards, and the others were deduced on the basis of accurate molecular weights and MS^2 fragments. IT-TOF-MS provides accurate mass measurements for both precursor-ions and fragmental ions with a precision of “mass errors < 5 ppm” so as to be convenient for identification and verification of chemical markers. Mass spectra in both positive and negative ionization mode were detected simultaneously in a full-scan operation in support of obtaining more comprehensive

structural information. The application of the same gradient elution program in both ESI+ and ESI- offers advantages in chemical identification, as a single compound will be recorded with the same retention time in both ion modes. The VIP value, *p* value, polarity and origin of 26 chemical markers were shown in supplementary Table 2.

3.5 Comparison of four decoctions

Fig. 3 illustrates the changes of the content of chemical markers in different groups in a box-plot. Twenty-six chemical markers screened showed different concentration in different groups. For HPSWD and HPDHD, the concentration of most constituents in twenty-six screened markers was similar which may be because of the same composition and similar proportions of them. Studies have found that rhein was the only anthraquinone aglycone absorbed by human body after oral administration of the boiling-water extract of *Radix et Rhizoma Rhei*, and the biotransformation product of rhein by human intestinal bacteria, rhein anthrone, had a strong purgative activity which was associated with reducing the absorption of sodium ions and chloride ions and increasing secretion of potassium ions by colon [23-24]. The maximum concentration of rhein in DCQD while the minimum concentration in XCQD was in accordance with the strongest laxative effect of DCQD and the weakest laxative effect of XCQD. Magnolol from *Mangnolia officinalis* exerted prokinetic and inhibitory effects on gastrointestinal movement and streptococcal glucosyltransferases respectively [25-26]. Magnolol also appeared higher content in DCQD which may be responsible for the stronger bacteriostatic activity of DCQD. Naringin, neohesperidin and hesperidin are bioactive flavonoids present in *Fructus Aurantii Immaturu*. Like most flavonoids, naringin, neohesperidin and hesperidin possessed antioxidant, anti-inflammatory and anti-ulcer properties [27-28]. The three flavonoids showed obviously different concentration in four decoctions may be responsible for different levels of anti-inflammatory and antimicrobial effects of them. The reason of diverse pharmacological effects of four multi-herb decoctions may be due to the different concentration of the twenty-six chemical markers which remains to be further investigated by comparative pharmacological and clinic studies.

4 Conclusion

In the present manuscript, an UFLC-IT-TOF-MS method combined with chemomic and chemometric approach was proposed to investigate the chemical differentiation of DCQD and its three analogous decoctions. Compared to traditional methods to quantification of several active constituents, the suggested method could comprehensively and integrally describe the chemical profiles of four prescriptions of TCMs, and effectively

discover the chemical markers. Our findings demonstrated that there were obvious chemical differences among the four decoctions. Twenty-six potential chemical markers were discovered, of which five were from the root and bark of *Radix et RhizomaRhei*, eight from the bark of *Magnolia officinalis Rehd* and the rest from *Fructus Aurantii Immaturus* after identification by retention time and MS data. The twenty-six screened chemical markers may be the reason for different pharmacological effects of four decoctions based on the fact that they revealed different concentration in these four decoctions. However, it remains to be further investigated by comparative pharmacological and clinic studies and this manuscript could also lay a foundation for the subsequent metabolomics study of the four decoctions *in vivo*.

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Table 1 Prescription of DCQD, XCQD, HPDHD, HPSWD and three single-herb decoctions

Decoctions	Radix et Rhizoma Rhei (g)	Magnolia officinalis Rehd (g)	Fructus Aurantii Immaturus (g)	Mirabilitum (g)
DCQD	12	24	15	6
XCQD	12	6	9	\
HPDHD	12	24	15	\
HPSWD	18	24	12	\
Radix et Rhizoma Rhei	12	\	\	\
Magnolia officinalis Rehd	\	24	\	\
Fructus Aurantii Immaturus	\	\	15	\

Table 2 Repeatability and precision (RSD) of QC samples in UFLC-IT-TOF-MS

markers	Repeatability (0h,6h,12h) (RSD %) (n=3)	intra-batch precision (RSD %) (n=5)	inter-batch precision (RSD %) (n=15)
catechin	5.48	5.51	5.92
rhein	4.69	1.81	6.78
magloside A	6.44	3.08	7.05
magnolol	4.73	4.87	7.93
naringin	5.87	2.88	5.97
neohesperidin	3.85	5.20	3.40

Table 3 Components identified from DCQD, XCQD, HPDHD, HPSWD

Peak no.	polarity	t_R(min)	Assigned identity	Molecular formula	Measured mass (Da)	Theoretical exact mass (Da)	Mass accuracy (ppm)
1	−	0.915	Scopoletin[b]	C10H8O4	192.04133	192.04226	0.93
2	+	1.018	Synephrine[a]	C9H13NO2	167.09137	167.09463	3.26
3	−	1.216	3,5-dihydroxy phenyl 1-O-β-D-glycosidase[b]	C12H16O8	288.08294	288.08452	1.58
4	−	1.267	Citric acid[b]	C6H8O7	192.02693	192.02701	0.08
5	+	1.553	Adenosine[b]	C10H13N5O4	267.0975	267.09676	-0.74
6	+	5.582	Catechin[a]	C15H14O6	290.07387	290.07904	5.17
7	+	10.117	Cinnamoyl glucose[b]	C15H18O7	310.10413	310.1053	1.17
8	−	10.535	Magnoloside A[b]	C29H36O15	624.20093	624.20542	4.49
9	+	12.033	Cinnamoyl glucose[b]	C15H18O7	310.10453	310.1053	0.77
10	−	12.393	Neoeriocitrin[b]	C27H32O15	596.16983	596.17413	4.3
11	−	13.964	Naringin[a]	C27H32O14	580.17643	580.17921	2.78
12	−	14.348	Magnolol B[b]	C18H20O5	316.13063	316.13108	0.45
13	−	14.592	Acteoside[b]	C29H36O15	624.20163	624.20542	3.79
14	−	14.596	Hesperitin-glucoside[b]	C22H24O11	464.13053	464.13186	1.33
15	+	14.781	Hesperidin[a]	C28H34O15	610.18983	610.18978	-0.05
16	−	14.906	Magnoloside E[b]	C28H34O5	610.18773	610.18978	2.05
17	−	14.947	Neohesperidin[a]	C28H34O15	610.18773	610.18978	2.05
18	−	15.445	Rhoifolin[b]	C27H30O14	578.15983	578.16356	3.73
19	+	18.064	Limonin[b]	C26H30O8	470.19057	470.19407	3.5
20	−	18.603	Magnolignan A[b]	C18H20O4	300.13533	300.1362	0.87
21	+	21.398	Marmin[b]	C19H24O5	332.15713	332.16238	5.25
22	−	21.456	Magnaldehyde D[b]	C16H14O3	254.09173	254.0943	2.57
23	−	22.689	Magnaldehyde B[b]	C18H16O3	280.10853	280.10995	1.42
24	−	23.757	Rhein[a]	C15H8O6	284.03133	284.0321	0.77
25	+	24.063	Tangeretin[b]	C20H20O7	372.11617	372.1209	4.73
26	−	24.633	Magnolol[a]	C18H18O2	266.12913	266.13068	1.55

a: markers were identified by comparing with standards, b: markers were deduced on the basis of accurate molecular weights and MS^2 fragments

Figure captions:

Fig.1 Representative base peak intensity (BPI) chromatograms of DCQD. BPI chromatograms were monitored in both positive (A) and negative (B) ion mode

Fig.2. Scores plots of PCA (A:ESI+; B:ESI-) and OPLS-DA (C:ESI+; D:ESI-) models of the peaks of *Mangnolia officinalis* with the statistical parameters as follows: A R^2X=0.701, Q^2=0.294; B R^2X=0.713, Q^2=0.342; C R^2X=0.854, R^2Y=0.981, Q^2=0.709; D R^2X=0.586, R^2Y=0.845, Q^2=0.597

Fig. 3. The box-plot showed different concentration of chemical markers in different groups for A: chemical markers screened from *Rheum officinale*. B: chemical markers screened from *Mangnolia officinalis*. C: chemical markers screened from *Fructus Aurantii Immaturus*

Fig.1

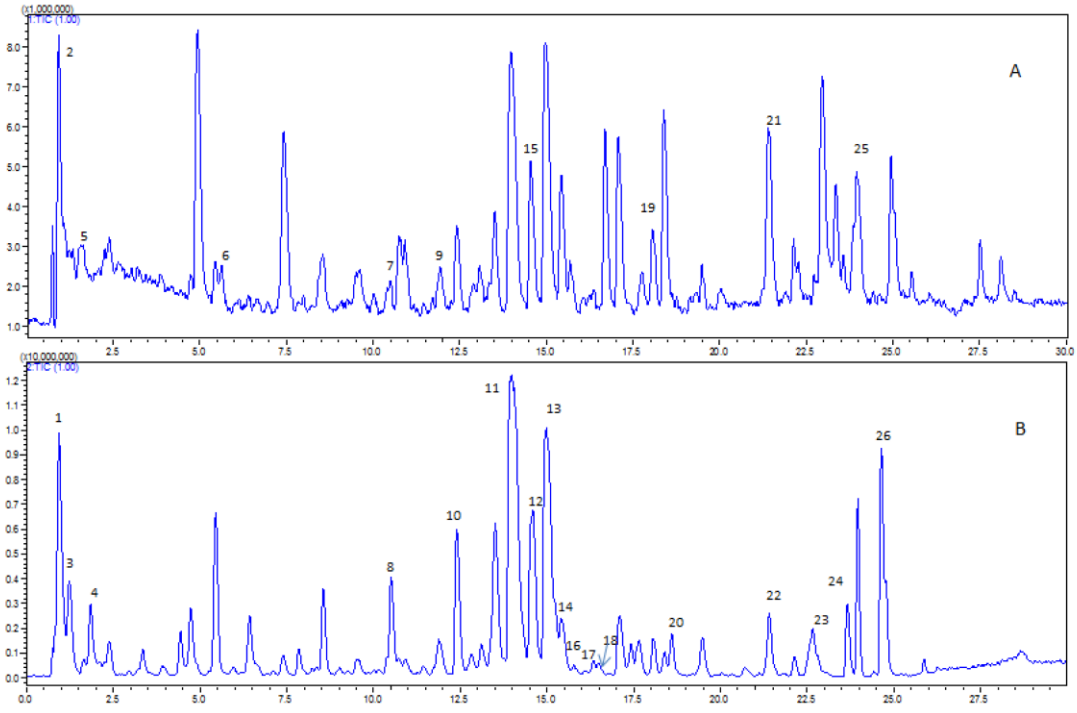

Fig.2

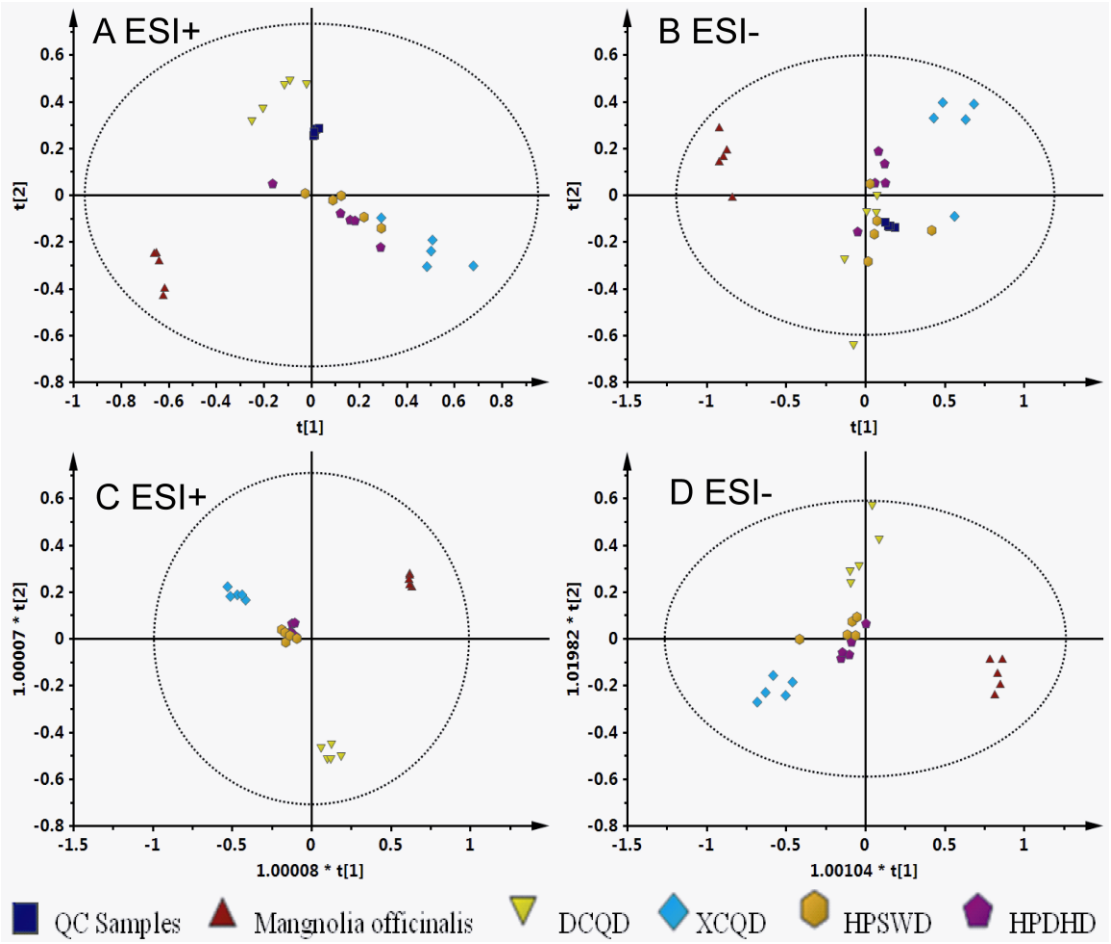

Fig.3