Influence of different processing times on the quality of *Polygoni Multiflora Radix* by metabolomics based on ultra high performance liquid chromatography with quadrupole time-of-flight mass spectrometry

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Running title: Influence of different processing times on *Polygoni Multiflora Radix*

Standard Abbreviation: FDA, Fisher′s discriminant analysis; PMR, *Polygoni Multiflora Radix*; PCA principal component analysis; PLS-DA, Partial Least Squares Discriminant Analysis; VIP, variable importance parameters

Keywords: Fisher's discriminant analysis; metabolomics;*Polygoni Multiflora Radix*; principal component analysis; traditional Chinese medicine

Abstract

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A metabolomics method based on ultra high performance liquid chromatography with quadrupole time-of-flight mass spectrometry was developed to evaluate the influence of processing times on the quality of raw and processed *Polygoni Multiflora Radix*. Principal component analysis and partial least squares discriminant analysis was used to screen the potential maker metabolites that were contributed to the quality changes. Then these marker metabolites were selected as variables in Fisher's discriminant analysis to establish the models that were used to distinguish the raw and processed *Polygoni Multiflora Radix* in the markets. Additionally, 36 compounds were identified. 12 raw *Polygoni Multiflora Radix* samples and 23 processed *Polygoni Multiflora Radix* samples were distinguished. The results showed that the 12 raw *Polygoni Multiflora Radix* samples belonged to the group of processing time of 0 h, and two processed *Polygoni Multiflora Radix* samples were part of the group of processing times of 4 h, 12 samples belonged to group of processing times of 8 to 16 h, and nine samples were the group of processing times of 24 to 48 h. The results demonstrated that the method could provide scientific support for the processing standardization of *Polygoni Multiflora Radix*.

1. Introduction

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Traditional Chinese medicines (TCMs) have been used for the prevention and treatment of a variety of diseases for quite a long time. At present, TCMs have gained more and more attention with the increased awareness of healthcare among people. According to the TCM theory, raw materials sometimes need to be processed by heating, steaming and soaking to enhance efficacy or reduce toxicity before they were used in the TCM prescriptions [1]. Nowadays, many TCM and their processed products are commercially available in the markets of China.

Polygoni Multiflora Radix (Heshouwu in Chinese, PMR) was one of widely used TCMs, which was used for the treatment of hyperlipidemia, heart disease and other diseases associated with aging [2–3]. The main components in PMR consist of stilbenes, anthraquinones, phenolicacids, flavonoids and their glycosides [4–5]. Studies have shown that stilbenes have the anti-aging, anti-inflammatory, anti-oxidative and liver-protective activities [6–7]. Anthraquinone possessed the activities of anti-bacterial, anti-fungal, antioxidant and anti-cancer [8]. Phenolic acids and flavonoids exhibited the antioxidant activity in vitro and in vivo [9]. It was these components contribute to the pharmacological activities of PMR. According to Chinese Pharmacopoeia, the raw *Polygoni Multiflora Radix* (R-PMR) was processed by steaming with black bean juice, which was named as the

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processed *Polygoni Multiflora Radix* (Zhishouwu in Chinese, P-PMR). Previous reports showed that the contents of some compounds were enhanced or decreased during the processing of R-PMR [10]. The changes might be responsible for the changes of the pharmacological effects of R-PMR and the hepatotoxicity [11]. Since the processing of TCMs could result in the components changes and the changes could result in different pharmacological activities, it is necessary to characterize the processing state of PMR for more safe and efficacious use. Currently, R-PMR and P-PMR are of commercially availability in the herbal markets. However, there is no exact standard to abide for the processing of PMR. Thus, it is urgent to develop a method to establish a processing standard for the QC of PMR in the markets.

Metabolomics is considered as a systematic approach to deal with parallel assessment of the levels of a variety of metabolites and have played an important role in both phenotyping and diagnostic analyses in plants [12]. These methods have been applied for the assessment of natural variance in metabolite composition [12]. Nowadays, modern analytical techniques like HPLC–MS, UHPLC–MS and GC–MS are used in metabolomics studies [13]. Among the analytical techniques in metabolomics researches, UHPLC–MS is taken as one of the best analytical methods with high sensitivity, selectivity and reproducibility [14–15]. Moreover,

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the combination of metabolomics techniques with chemometric method could provide a fast reliable method for the biomarker screening, component identification and QC of TCMs [16].

In the study, a simple and reliable UHPLC–Q-TOF-MS-based metabolomics method was developed and validated to evaluate the influence of different processing times on the quality of PMR. Furthermore, principal component analysis (PCA) was used to select influential biochemical markers for distinction of the sample groups. The Fisher′s discriminant analysis (FDA) was applied to establish the standard models by using the screened biochemical markers in the different processing times of PMR samples. Finally, the discriminant models were used to distinguish the PMR in the markets to standardize the process of PMR. That is, supervised and unsupervised methods of data analysis were used to discriminate PMR samples with different processing times. Overall, the Q-TOF-MS-based metabolomics approach could be used to provide general quality evaluation of PMR in the markets.

2. Materials and Methods

2.1 Plant materials

R-PMR and P-PMR samples were purchased from Tianjin pharmacy and authenticated by Dr. Yan-xu Chang (Tianjin University of Traditional Chinese Medicine). The voucher

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specimens were deposited at Tianjin University of Traditional Chinese Medicine, Tianjin, China.

2.2 Chemicals and reagents

Acetonitrile was purchased from Merck (Darmstadt, Germany) and methanol of HPLC grade was obtained from Tianjin Concord Science (Tianjin, China). Formic acid of HPLC grade was purchased from Tedia (Fairfield, OH,USA). Deionized water was purified with a Milli-Q Academic ultra-pure water system (Millipore, Milford, MA, USA). Reference Standards of gallic acid, catechin, epicatechin, 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside, resveratrol, emodin-8-*O*-glucoside, physcion-8-*O*-glucoside, rhein and emodin (purity > 98%)were purchased from Chengdu Must Biotechnology (Chengdu, China). Other reagents were of analytical grade and obtained commercially.

2.3 Sample preparations

2.3.1 Preparation of P-PMR extract

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P-PMR was processed by black soybean decoction according to Chinese Pharmacopoeia. The processing procedure was as follows: 2 kg R-PMR was mixed with black beans extract (0.2 kg black beans were extracted with some water for 4 h for 0.3 kg decoction, then the soybean dregs were continued to be boiled by water for 3 h for 0.2 kg decoction. Finally 0.5 kg black soybean decoction were obtained) and then steamed in the steamer by boiling water for different times. Finally the P-PMR samples of different times at 4, 8, 12, 16, 24, 32, 40 and 48 h were obtained. The raw RPM was named as the P-RPM samples at 0 h processing time.

The dried powder of R-PMR and P-PMR (0.1 g) samples were weighed accurately and extracted with 10 mL 70% v/v methanol ultrasonically for 20 min. After centrifugation at 14 000 rpm for 10 min, the supernatants were filtered through 0.22 μm filter [5]. Then the extracts of R-PMR and P-PMR which was prepared with different processing times were obtained.

2.3.2 Preparation of standard solutions

Gallic acid, catechin, epicatechin, 2,3,5,4-tetrahydroxystilbene-2-*O*-β-D-glucoside, resveratrol, emodin-8-*O*-glucoside, physcion-8-*O*-glucoside, rhein and emodin with the

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concentration of 1.0 mg mL^{-1} were prepared in methanol. The reference standards solution was diluted serially to achieve the standard working solutions.

2.4 UHPLC–Q-TOF-MS analysis

Aglient 6520 Q-TOF mass spectrometer (Agilent Corporation, Santa Clara, CA, USA) coupled with the Agilent 1290 UHPLC by an ESI interface was used to identify the components in PMR extract. The chromatographic and ESI-MS spectra conditions were used to separate and identify the markers and components in sample according to our previous study [17].

2.5 Method validation

The method validation included precision, repeatability and stability. The mixed standard solutions were used for the method validation. The precision was investigated by one sample with six replicate injections. The repeatability of the method was assessed by performing six replicate solutions. The stability of those analytes was assessed by analyzing the solution at 0, 2, 4, 6, 8, 12 and 24 h. The validation was expressed as the RSD.

2.5 Data Analysis

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For the metabolite profiling of R-PMR and P-PMR, the UHPLC–Q-TOF-MS data were analyzed by the XCMS software operating on the R^+ package (R Foundation for Statistical Computing, Vienna, Austria). The intensities of detected peaks were tabulated using tR-*m/z* pairs and exported for statistical analyses. Data processing, which included handling missing values and normalizing the data set, was performed to convert the data into the proper data sets [18–19]. After data processing, chemometrics methods were applied to the data sets to select influential metabolites for discrimination of the sample groups. All processed data were analyzed using principal component analysis (PCA) to discriminate raw and processed PMR in the TCM markets and select influential metabolites. Partial Least Squares Discriminant Analysis (PLS-DA) was used to test the classification performance of discriminant the P-PMR samples of different processing times. Then the selected metabolites were applied in the Fisher′s discriminant analysis (FDA) for the establishment of the discriminant models by the P-PMR samples of different processing times. Finally the models were used to classify the PMR samples in the markets. The PCA and PLS-DA were analyzed by Simca P version 11.5 (Umetrics, Umea, Sweden) and FDA was analyzed by SPSS version 19.0 (SPSS, Chicago, IL, USA) for data analysis.

3. Results

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3.1 Optimization of UHPLC–Q-TOF-MS analysis conditions

The UHPLC–Q-TOF-MS analytical method was developed for the better resolution and detection of a wide range of metabolites in the PMR. The optimization of mass conditions was performed in negative ion mode for this ion mode provided more information on analyzing the PMR extract. Solvent systems including acetonitrile/water and methanol/water in different proportions and gradient durations were tried. As a result, acetonitrile/water containing 0.1% formic acid was selected as mobile phases. What′s more, other chromatographic conditions, like columns, column temperatures and flow rates were also optimized. Among the several conditions tested, an ACQUITY UHPLC BEH C_{18} (1.7 μm, 2.1×50 mm) column at column temperature of 30°C reached the better performance. The flow rate was set at 0.3 mL min^{-1}. A representative chromatogram of PMR is shown in **Fig. 1A**.

3.2 Method validation

To validate the developed method, the precision, repeatability and stability of the metabolite profiling study was carried out. Nine representative reference standards were used for the method validation. The results were listed in **Table 1**. As shown in **Table 1**, the RSD values

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of precision were less than 3.0%, indicating that the method was precise for the qualitative analysis of PMR extract. The results of the repeatability were no more than 2.82%, which demonstrated that the method was reproducible for compound identification. The RSDs of the stability of the analytes were less than 3.27%, demonstrating the sample solutions were stable with 24 h at room temperature. The above results demonstrated that UHPLC–Q-TOF-MS method could be used for the metabolite profiling study of PMR extract.

3.3 Multivariate statistical analysis

We analyzed 12 R-PMR and 23 P-PMR samples by the UHPLC–Q-TOF-MS method. All chromatographic information obtained from the metabolite profiling of 35 PMR samples were analyzed by the XCMS software. A three-dimensional data matrices containing sample information, variables characterized by retention time and *m/z* value as well as their corresponding intensities were obtained and exported to an Excel table. The data were preprocessed following the method described previously [20]. Moreover, the *p* values in T-test also were given. Totally 1256 processed and treated metabolites with *p* values less than 0.05 were selected out for the PCA analysis to determine the similarities and differences

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among the 35 PMR samples. It was not observed that the new components were obviously produced during the processing. As shown in **Fig. 2**, the three-dimensional PCA score plot showed a fairly clear differentiation between R-PMR and P-PMR.

To select influential metabolites for the discrimination, the datasets were applied to the statistical classification method. As a result, 35 metabolites (M115, M121, M128, M133, M169, M179, M186, M191, M195, M215, M269, M270, M278, M283, M289, M290, M341, M377, M379, M389, M404, M407, M419, M431, M439, M440, M441, M511, M517, M564, M683, M684, M811, M813 and M863), the key constituents for the discrimination of R-PMR and P-PMR, were selected from 1256 metabolites. The results were shown in **Fig. 3**. Furthermore, PLS-DA model was used to select key markers from these 35 metabolites. This stems from the fact that it can select markers depending on variable importance parameters (VIP > 1) which can then be used to be selected as markers according to the order of their contributions to the separation of clustering. Based on the VIP, 15 metabolite markers can be screened. The results are shown in **Fig.3**. It was illustrated that 15 metabolite markers were regarded to be the components which contributed most to discrimination of R-PMR and P-PMR.

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3.3 Compound identification in PMR extract

The identification of the components in PMR extract was carried out by UHPLC–Q-TOF-MS. The 70% methanol extract of PMR was employed to obtain the total ion chromatogram (TIC) of MS study and MS/MS study of the fragment ion. As can be seen from **Table 2**, 36 compounds were identified or characterized tentatively according to previous reports

3.3.1 Identification of gallates and tannins

Seven gallates and tannins were detected, including gallic acid and monomers, dimmers and trimers of catechin/epicatechin units. The identification of tannins was conducted by comparing the MS/MS fragmentation information with previous reports [21–22]. MS/MS spectra of negative mode produced abundant ions were listed in **Table 2**. Compounds **2**, **4**, **5** and **13** were identified unambiguously by comparing with the reference compounds. They were identified as gallic acid, catechin, epicatechin and catechin gallate, respectively [17]. The component 35 was identified by extracting ion at *m/z* 577 from TIC. The precursor ion at *m/z* 577 ($C_{30}H_{25}O_{12}$) was founded in MS spectra and most abundant ion at *m/z* 425 was given in MS/MS spectrum. The fragment ions at *m/z* 289 and 287 could be attributed to the

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quinonemethide fission cleavage of the type-B interflavan bond resulting from the loss of an epi/catechin residue. As a result, it was identified as procyanidin B by comparing the MS information with the literature [23]. A predominant peak of compound 29 was screened out by parents scan for ion at *m/z* 289. Compound **29** was identified as a trimer of catechin for its characteristic fragment ions of *m/z* 577 and *m/z* 289, which were the successive losses of catechin unit[24]. In addition, another trimerepi/catechin was detected by selecting the corresponding high-resolution $[M–H]^-$ precursor ions in negative mode. Thus, compound **30** was identified as prodelphinidin tentatively [23].

3.3.2 Identification of stilbenes

2,3,5,4′-Tetrahydroxystilbene-2-*O*-β-D-glucoside (**11**, THSG) gave a $[M–H]^-$ ion at *m/z* 405 and $[2M–H]^-$ at 811 in the negative mode. In the MS/MS spectrum, THSG gave a dominant ion at *m/z* 243, which could be recognized as the diagnostic ion for stilbenes. Further fragmentation produced four most abundant ions at *m/z* 225, 215, 149 and 137. Compound 1 gave a $[M–H]^-$ ion at *m/z* 403 in MS spectrum and prominent fragment ion at *m/z* 241 in MS/MS spectrum, which were the loss of two glc units from THSG. Furthermore, the loss H_2O and CO of fragment ion at *m/z* 241 formed the ions at 223 and 213, respectively. The

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consecutive loss of CO produced ions at *m/z* 195 and 169. Compound **1** was identified as tetrahydroxy-phenanthrene -*O*-hexoside preliminarily [23]. Compound **9** gave a $[M–H]^-$ ion at *m/z* 567 in the MS spectrum. The consecutive loss of hexoside form edionsat *m/z* 405 and 243 in MS/MS spectra. It was characterized astetrahydroxystilbene-*O*-dihexoside by comparing previous report [25–26]. Compounds **14** and **15** have the same $[M–H]^-$ ion at *m/z* 557 in MS spectra and identical ions at *m/z* 313, 405 and 243 in MS/MS spectra. The ion at *m/z* 405 was produced by the loss of $C_7H_4O_4$ and ion at *m/z* 313 produced gallic acid deprotonated ion at *m/z* 169. By comparing the information with literatures, compounds **14** and **15** were identified as astetrahydroxystilbene-*O*-(galloyl)hexosides [27]. Compound **17** showed the $[M–H]^-$ ion at *m/z* 421 and $[M+HCOO]^-$ ion at *m/z* 467 in MS spectra. Owing to the hexose loss, the prominent ion at *m/z* 259 was produced. Compared with the previous report, compound **17** was characterized as pentahydroxystilbe neglucoside tentatively [26]. Compound **20** gave an ion of $[M–H]^-$ at *m/z* 551 and characteristic ion at *m/z* 243 was produced by the losses of $C_9H_6O_2$ and $C_6H_{10}O_5$ in MS/MS spectra. A minor ion at *m/z* 307 was also observed. As a result, compound **20** was identified as tetrahydroxystilbene-*O*-(coumaroyl) hexoside tentatively [28]. For the detection of loss of feruloyl moiety $C_{10}H_8O_3$, compound **19** was tentatively characterized as

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tetrahydroxystilbene-*O*-(feruloyl) hexoside similar to compound **20** [29]. Compound **10** was identified as polydatin according to previous research [17,26]. Compound **18** was identified as resveratrol by comparing with the reference standard.

3.3.3 Identification of anthraquinones

Physcion (**34**) showed the $[M–H]^-$ ion at *m/z* 283 and yielded the ion at *m/z* 268 for the loss of CH_3 free radical. The ions at *m/z* 240, 212 and184 were produced by the further elimination of CO. Emodin (**33**) gave an $[M–H]^-$ ion at *m/z* 269, which formed two prominent ions at *m/z* 225 and 241 by the loss of a CO and CO_2, respectively. Two ions at *m/z* 181 (loss of a CO_2) and 210 (loss of a methyl free radical) were determined from the ion of *m/z* 225. The ion at *m/z* 197 was generated by the loss of $CO_2$from ion at *m/z* 241. Chrysophanol (compound **36**) showed the $[M–H]^-$ ion at *m/z* 253 in the negative mode and prominention at *m/z* 225 due to loss of CO in MS/MS spectra. Ions at *m/z* 210 and 181 were resulted from the losses of a methyl radical and CO_2 molecule of the ion at *m/z* 225. Aloe-emodin (**7**) has the same $[M–H]^-$ ion at *m/z* 269 and molecular composition with emodin. The ion at *m/z* 240 was generated by the loss of CHO from *m/z* 269 in the MS/MS spectra, from which generated the ion at *m/z* 211 by the loss of CHO. Physcion-8-*O*-glucoside (**25**), emodin-8-*O*-glucoside (**22**),

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rhein (**31**) and citreorosein (**24**) were identified by comparing the chemical information with reference standard compounds. Compound **23** gave a $[M–H]^-$ ion at *m/z* 517. The malonyl substituent on the glycosyl residue was deduced by further elimination of CO_2 in MS/MS spectra. It was identified as emodin-*O*-(malonyl) hexoside by comparing the MS data with that of a previous report [23]. Compound **28** showed the $[M–H]^-$ ion at *m/z* 473 and an ion at *m/z* 311 by the loss of hexose unit in MS/MS spectra and then produced the ion at *m/z* 269 for further fragmentation. Compound **28** was likely to be acetylemodin-*O*-hexoside [23]. Compound **26** exhibited a $[M–H]^-$ ion at *m/z* 473 in MS spectra and ions at *m/z* 377 and 269 in MS/MS spectra. By comparing the MS data with literature, compound **26** was identified as aloe-emodin-8-*O*-(6`-*O*-acetyl)-glucoside tentatively [29]. Compound **8** gave a $[M–H]^-$ ion at *m/z* 531 in the full scan of PMR extract. The ion at *m/z* 487 and 283 was produced by the loss of CO_2 and malonylglucosyl from the precursor ion in MS/MS spectra, respectively. Moreover, the ion at *m/z* 239 was got by the loss of CO_2 from the ion at *m/z* 283. As a result, compound **8** was identified as physcion-8-*O*-(6'-*O*-malonyl)glucoside according to the literature [30].

3.3.4 Identification of naphthalenes

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As far as we know, torachrysone-8-*O*-glucoside (**12** and **21**) was the only naphthaleneglycoside in PMR [23]. Torachrysone-8-*O*-glucoside, the $[M–H]^-$ ion at *m/z* 407 lost one glucosyl residue to generate the prominent ion at *m/z* 245 and then eliminated CH_3 free radical to produce the ion at *m/z* 230. For acetyl torachrysone glycoside, the ion at *m/z* 245and characteristic ions at *m/z* 230 and 215 by loss of two CH_3 free radicals was observed. Thus compound **32** was identified tentatively as torachrysone-*O*-(acetyl)hexoside [31]. Compound **6** showed the $[M–H]^-$ ion at *m/z* 393 in the MS spectrum and then eliminated a hexose unit to produce the ion at *m/z* 231. The ion at *m/z* 231 continued to lose the CO_2 and CH_2CO to generate the ion at *m/z* 187 and 189, respectively. As a result, it was identified as hydroxymusizin-*O*-hexoside tentatively [23].

3.3.5 Identification of other compounds

Apart from the identified compounds above, several other compounds (**3**, **16** and **27**) were also identified in the study. Their chemical information are listed in **Table 2**.

3.4 Structural study of selected marker metabolites

From the PCA results above, 35 maker metabolites(M115, M121, M128, M133, M169, M179, M186, M191, M195, M215, M269, M270, M278, M283, M289, M290, M341, M377,

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M379, M389, M404, M407, M419, M431, M439, M440, M441, M511, M517, M564, M683, M684, M811, M813 and M863) were selected out for the discrimination of R-PMR and P-PMR. The results were listed in **Table 3**. Among the 35 maker metabolites, 15 compounds were identified according to our results of qualitative analysis. The other 20 compounds were still unknown and our identification work will continue.

3.5 Discriminant analysis

In the study, unsupervised principal component analysis (PCA) was firstly applied to investigate the known 29 P-PMR samples at different processing times (0, 4, 8, 12, 16, 24, 32, 40 and 48 h) according to 15 maker metabolites. As shown in **Fig.4A,** under the unsupervised model, 29 P-PMR samples at different processing time can be preliminary divided into four different groups depending on distribution property, which was illustrated that P-PMR samples with different processing time at 0, 4, 8–16 and 24–48 h can be clustered. After prediction, the discriminant analysis was used to build the predictive model of the group membership based on observed characteristics of variables. It produced a discriminant function (for more than two groups, a set of discriminant functions) based on the predictor variables that provide the discrimination among the groups. The discriminant

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functions were generated by the samples with known groups. Then discriminant functions were used to predict the predictor variables with unknown groups.

Here, 29 P-PMR samples were collected at different processing times (0, 4, 8, 12, 16, 24, 32, 40 and 48 h), and PLS-DA [32–33] model was applied again to validate whether they could be separated according to 35 markers and 15 markers. As obviously shown in **Fig.4B** and **Fig.4C**, 4 groups (0 h to one group, 4 h to one group, 8 to 16 h to one group and 24 to 48 h to the last group) were separated well depending on 15 markers as same as using 35 markers. Therefore, 15 metabolite makers can be used as variables for the establishment of the discriminant function. However, not all variables could be used for the establishment of the discriminant function. Only the valuable predictor variables were of importance to the generation of the discriminant functions. After the discriminant analysis using the SPSS software, only 14 variables were selected for the establishment of the discriminant function. The four discriminant functions of PMR generated from the different processing times were as follows:

Group1=0.000065885295x1+0.000086776115x2–0.000000756662x3–0.000157974117x4–0.001301271997x5+0.000046073611x6–0.000024018062x7+0.000042095163x8–0.000673736

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933x9+0.000004711657x10–0.000042310637x11+0.000283186084x12+0.000006733512x13–0.000029951111x14–518.485421295047

Group2=0.00004582315x1+0.000132116003x2–0.00014813681x3–0.000157679338x4+0.000624415946x5+0.000071567204x6–0.000039769481x7+0.000100790637x8–0.001579335678x9+0.000002449869x103–0.000046686166x11+0.000337252255x12+0.000002219651x13–0.000036448989x14–696.409882440827

Group3=0.000050911879x1+0.000064893671x2–0.000026210477x3–0.000101949668x4–0.000689318802x5+0.000033893287x6–0.000021532886x7+0.000041543332x8–0.000488599467x9+0.000003184608x10–0.000034489071x11+0.000230166438x12+0.000004439329x13–0.000022758896x14–297.312167291817

Group4=0.000068735928x1+0.00008846915x2–0.000048268874x3–0.00011361905x4–0.001528276717x5+0.000027687492x6–0.000014014032x7+0.000018717914x8–0.00085575779x9+0.000004890566x10–0.000031915674x11+0.00023368023x12+0.000008409557x13–0.000018287617x14–376.383930338424 where Group 1 denotes samples of 0 h, Group 2 denotes samples of 4 h, Group 3 denotes samples of 8 to 16 h and Group 4 denotes samples of 24 to 48 h; x1 to x14 represents M133, M169, M179, M215, M278, M341, M377, M379,

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M389, M404, M439, M440, M517, M683. Finally, the classification result showed that 100% of originally grouped cases were correctly classified and 95.1% of cross-validation grouped cases were further correctly classified. The above results demonstrated that the discrimination model was reliable. The samples belonged to the group where the calculated value of the functions was the highest. The detailed data of four different discriminant function scores of 29 samples are shown in **Table.4.**

From the discriminant functions, only 14 variables were used to produce the functions. 35 PMR samples (12 R-PMR and 23 P-PMR) from different pharmacies in Tianjin were distinguished by the discriminant functions. The values of the 15 variables were put into the four discriminant functions to describe which group the 35 samples was classified into. The samples belonged to the group where the calculated value of the functions was the highest. The results demonstrated that the R-PMR and P-PMR samples from different pharmacies in Tianjin were clustered into the group of processing times of 0 and 4–48 h, respectively, further illustrating that the models established by the discriminant analysis were accurate. As far as the P-PMR samples are concerned, they were divided into three parts. The detail results of prediction of 35 samples are shown in **Table.5**. Two P-PMR samples were clustered into the group of processing time of 4 h, 12 samples belonged to the group of 8–16 h and nine

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samples were divided into the group of 24–48 h. The results demonstrated that the established discriminant models could be used to standardize the processing of PMR in the market.

To show the regular change of P-PMR samples with difference processing time, it was essential to comparing the content of screening 14 markers in different groups. **Fig.1B** showed the total ion chromatogram (TIC) of 14 markers in the four groups. As shown in **Fig.5**, the average intensity of M133, M179, M215, M278, M389 and M517 improved with the processing times 0–48 h, after which the contents showed no regular changes. With the increasing time of processing, the average intensity of M341, M377, M379, M404, M439, M440 and M683 were decreased. However, M169 (gallic acid) presented a completely opposite tendency. The content of gallic acid was increased as the processing times increased. As a result, processing could affect the contents of components in PMR and pharmacological effects of PMR, which need to carry out a deep research on this phenomenon.

4 Concluding remarks

The UHPLC–Q-TOF-MS-based metabolomics method was developed and validated for the evaluation of the influence of different processing times on the quality of PMR. Principal component analysis and Partial Least Squares Discriminant Analysis was successfully

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applied to screen the 35 maker metabolites, which were used to establish the discriminant models in the Fisher's discriminant analysis. With the approach, different PMR samples, which are commercially available in market, could be precisely classified using the detected metabolites. The R-PMR samples and P-PMR samples could be distinguished. The results showed that the 12R-PMR samples belonged to the group of processing time of 0 h while two processed PMR samples were part of the group of processing times of 4 h, 12 samples belonged to group of processing times of 8–16 h and nine samples were part of the group of processing times of 20 to 48 h. The results confirmed the validity of the metabolite profiling study. Consequently, the method could help in the precise authentication of PMR and could also be applied for the processing standardization of PMR in the markets.

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Figure legends:

Fig 1.The total ion chromatogram (TIC) of the main components in *Polygonum multiflorum* extract (A) and the representative chromatograms of the 4 groups. B1(R-PMR processed for 0

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h), B2 (P-PMR processed for 4 h), B3(P-PMR processed for 8–12 h) and B4(P-PMR processed for 16–32 h).(M1-M14 present M683, M684, M377, M379, M404, M133, M169, M179, M389, M439, M440, M341, M278 and M215).

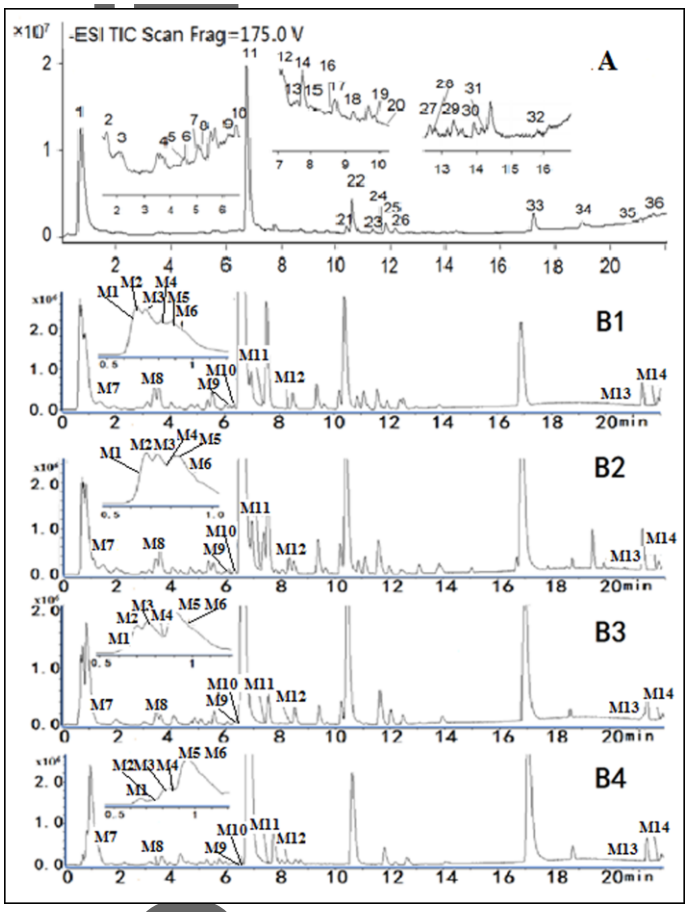

Fig 2. The score plot of the PCA of samples based on the 1256 variables with $p<0.05$.

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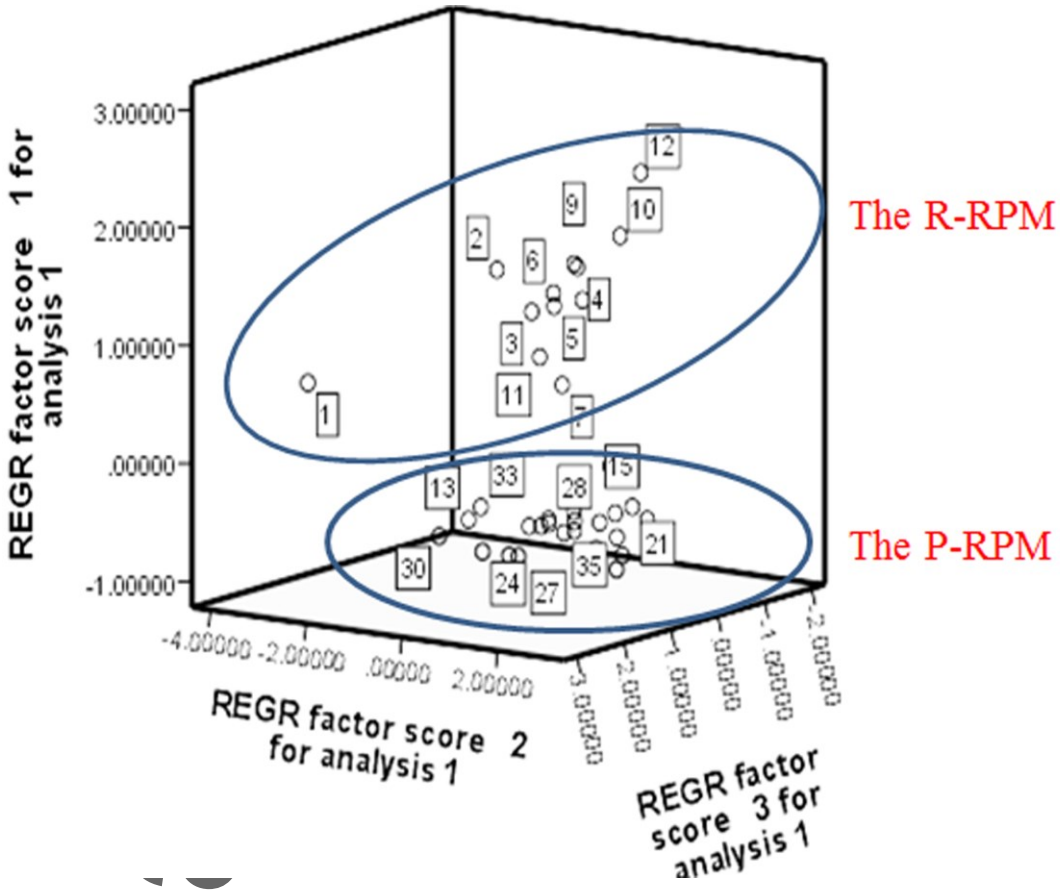

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Fig.3. The results of screened marker metabolites and the Numbers present the molecular weight of metabolites and one circle presents a metabolite and the results of screened marker metabolites and the selected 15 marker metabolites according to VIP value (>1).

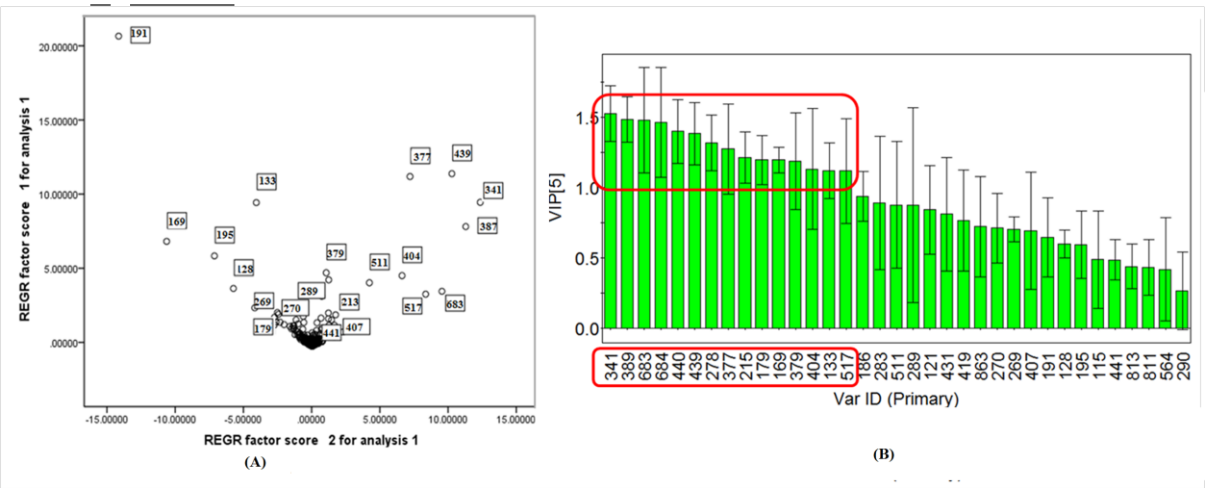

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Fig.4. PCA model for known 29 samples at different processing time (0, 4, 8, 12, 16, 24, 32, 40 and 48 h) and prediction for clustering another four groups (A); the PLS-DA model for 29 samples of four different processing times depending on 35 maker metabolites (B); the PLS-DA model for 29 samples of four different processing times depending on 15 maker metabolites(C).

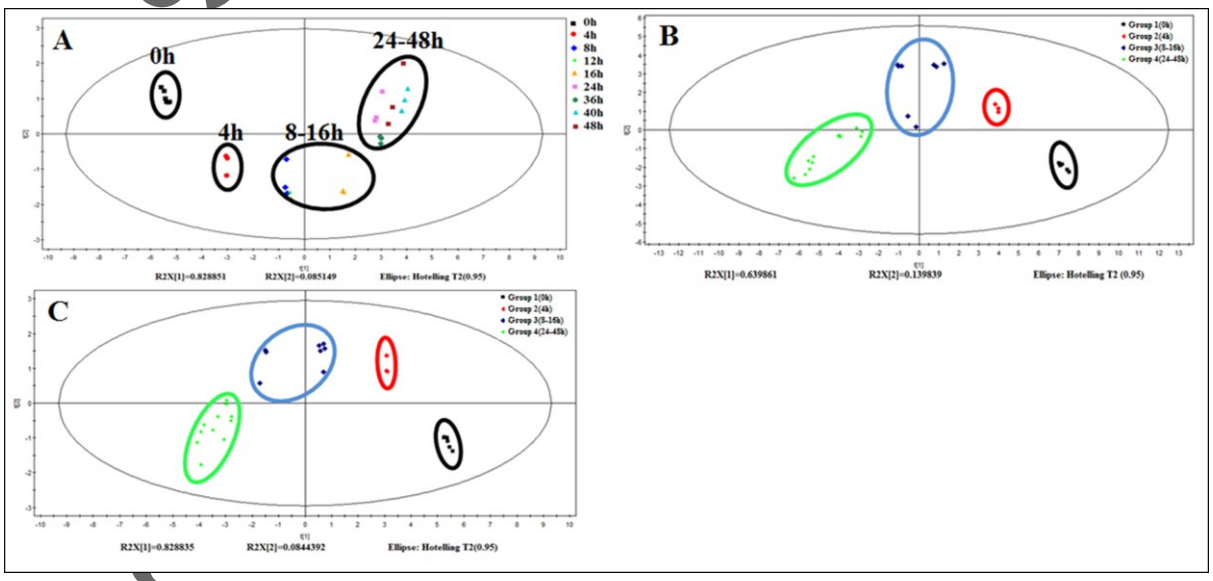

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Fig.5. The average intensity of 14 markers in the four different groups.

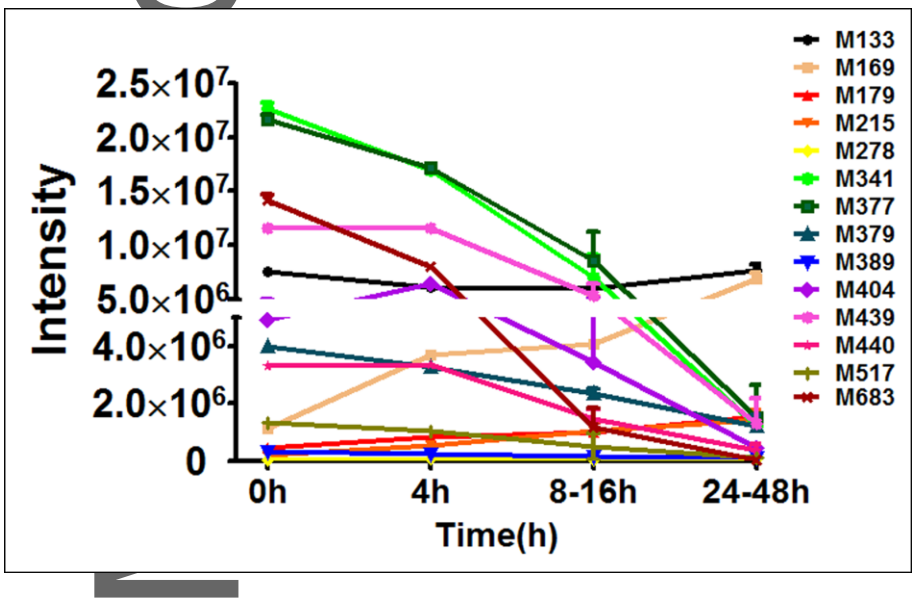

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Table 1 The method validation of UHPLC-Q-TOF/MS

Compound	Precision (RSD%)	Repeatability (RSD%)	Stability (RSD%)
gallic acid	2.32	1.91	2.56
catechin	3.00	2.40	2.49
epicatechin	1.83	2.82	2.56
PM-SG	1.10	2.63	2.79
resveratrol	2.41	2.64	3.27
emodin-8-*O*-glucoside	2.26	2.34	2.13

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physcion-8-*O*-glucoside	2.40	2.12	2.14
rhein	1.83	2.49	2.68
emodin	1.77	1.49	2.86

Table 2 The identification of compounds in R-PMR and P-PMR-extract

No.		[MS$^-$]	MS/MS	ppm	fo
1	0.786	403.1056	241.1022,213.1123,223.0221,195.0324,169.1231	0.21	C_{2}
2	1.409	169.0141		6.6	C
3	2.116	419.1653	213.1233,195.1119,128.0349,101.0712	1.5	C_{2}
4	3.725	289.0724	215.0714,173.0535,149.0206,125.0215,109.0283	2.2	C_{1}
5	4.48	289.0724	276.4677,205.0499,163.0368,131.0053,109.0317	2.2	C_{1}

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6	4.542	393.1603	231.1265,187.1096,189.0750	1.94	C_{1}
7	4.963	269.0120	240.0555,211.1021	3.89	C_{1}
8	5.081	531.1498	487.0979,283.0637,239.0802	1.88	C_{26}
9	6.227	567.1719	405.1198,243.0657,215.1021,149.0245	0.05	C_{26}
10	6.484	389.1168		3.89	C_{2}
11	6.748	811.0644	405.1180,243.0648, 225.3022,215.1024,149.2312,137.0237	1.03	C_{2}
12	7.145	407.1184	245.0656,230.0237,215.1011	0.95	C_{2}
13	7.574	441.0853	331.1021,289.0023,169.3201	2.31	C_{22}
14	7.728	557.1162	405.1142,313.0542,243.0645,169.0130	2.49	C_{27}
15	7.970	557.1034	405.1172,313.0550,243.0646,169.0125	2.49	C_{27}
16	8.433	121.0295	92.0254,65.0383	2.06	C
17	8.681	421.1146/ 467.0823	259.0563	1.43	C_{20}
18	9.242	227.2002	202.0698,176.0559,99.9223,91.0179,73.5614	3.35	C_{1}
19	9.865	581.1636	387.1131,331.0592,243.0638,405.0387	4.9	C_{30}

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20	10.202	551.1551	405.1134,307.0819,243.0658	0.57	C_{29}
21	10.472	407.1336	245.0800,230.0568,215.0348	0.12	C_{2}
22	10.597	863.2053/ 431.1386	269.0448,225.0540	0.86	C_{21}
23	11.324	517.1009	473.1057,269.0443	0.44	C_{24}
24	11.634	285.0380	257.0407,241.0483,211.0384,268.0361	0.7	C_{2}
25	11.786	445.0595	283.0595,240.0415	0.76	C_{21}
26	12.241	473.1089	377.0121,269.0453	0.48	C_{23}
27	12.596	329.2322	229.1418,211.13,183.1392,99.0808,57.0335	1.44	C_{1}
28	12.794	473.1124	311.1052,269.0477,207.8834,102.9316	1.02	C_{23}
29	13.339	865.2084	577.0965,289.0433	1.48	C_{42}
30	13.934	865.2053	577.0925,289.1403	1.48	C_{42}
31	14.127	283.0606	240.0409	3.76	C_{1}
32	15.879	447.1321	243.9898	4.4	C_{22}
33	17.188	269.0460	241.0491,225.0549,210.0312,197.0598,181.0122	3.89	C_{1}
34	18.939	283.0399	268.1120,240.0482,212.1121,184,0213	0.88	C_{1}
35	20.596	577.1357	425.0873,289.1143,287.0559	1.82	C_{30}

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36	21.417	253.2173	225.0423,210.0111,181.1011	1.9	C_{1}

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Table 3 The screened maker metabolites for the discrimination of R-PMR and P-PMR

No.	Marker	Rt.	MS	MS/MS	ppm	formula
1	M115	0.601	115.0022			
2	M683	0.669	683.2240			
3	M684	0.669	684.2269			
4	M377	0.702	377.0871	179.0354,119.0345,101.0233,89.0248,71.0136,59.0146		
5	M379	0.702	379.0831	179.0354,119.0345,101.0233,89.0248,71.0136,59.0146		
6	M404	0.786	404.1061	61.9891		
7	M133	0.801	133.0145			
8	M191	0.920	191.0205	57.0357,85.0304,87.0089,111.0076		
9	M169	1.409	169.0141		6.6	$C_7H_6O_5$
10	M128	1.796	128.0354	109.6663,103.2380,67.9247,60.9239		
11	M419	2.205	419.1682			
12	M186	3.464	186.0555	142.0656,116.0488		
13	M179	3.543	179.0549			
14	M289	3.725	289.0724	215.0714,173.0535,149.0206,125.0215,109.0283	2.2	$C_{15}H_{14}O$
15	M289	4.48	289.0724	276.4677,205.0499,163.0368,131.0053,109.0317	2.2	$C_{15}H_{14}O$
16	M195	5.724	195.0508			

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17	M389	6.484	389.1168		3.89	$C_{20}H_{22}O$
18	M439	6.748	439.0816			
19	M811	6.748	811.0644	405.1180,243.0648,225.3022,215.1024,149.2312,137.0237	1.03	$C_{20}H_{22}O$
20	M813	6.748	813.0644	405.1180,243.0648,225.3022,215.1024,149.2312,137.0237	1.03	$C_{20}H_{22}O$
21	M407	7.145	407.1184	245.0656,230.0237,215.1011	0.95	$C_{20}H_{24}O$
22	M440	7.574	441.0853	331.1021,289.0023,169.3201	2.31	$C_{22}H_{18}O$
23	M441	7.574	441.0853	331.1021,289.0023,169.3201	2.31	$C_{22}H_{18}O$
24	M121	8.433	121.0295	92.0254,65.0383	2.06	$C_7H_6O_2$
25	M341	8.714	341.1097	249.0698,89.0241,59.0141		
26	M511	9.655	511.0576	431.0976,121.0283		
27	M431	10.597	431.1386	269.0448,225.0540	0.86	$C_{21}H_{20}O$
28	M517	11.324	517.1009	473.1057,269.0443	0.44	$C_{24}H_{23}O$
29	M865	13.934	865.2053	577.0925,289.1403	1.48	$C_{42}H_{40}O$
30	M269	17.188	269.0460	241.0491,225.0549,210.0312,197.0598,181.0122	3.89	$C_{15}H_{10}O$
31	M270	17.188	270.0496	241.0491,225.0549,210.0312,197.0598,181.1021	3.89	$C_{15}H_{10}O$
32	M564	18.708	564.3323			
33	M283	18.939	283.0399	268.1120,240.0482,212.1121,184,0213	0.88	$C_{16}H_{12}O$

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34	M278	20.872	278.0894
35	M215	22.489	215.0327

Table 4. The 4 different discriminant functions scores of 29 samples

Sample name	Discriminant functions score				Classified
	Group1	Group2	Group3	Group4	
0h-1	525.5789	444.8304	473.3899	429.3605	Group1
0h-2	535.9555	450.1285	482.2046	450.8902	Group1
0h-3	523.0573	441.1327	471.8197	439.5416	Group1
0h-4	506.8386	416.3425	461.1772	428.2384	Group1
0h-5	506.4268	420.5351	459.7598	425.3151	Group1
0h-6	497.6219	410.4663	454.6204	417.8401	Group1
4h-1	620.1771	724.5048	568.5795	537.1318	Group2
4h-2	576.3855	658.6562	531.7202	500.3762	Group2
4h-3	613.4444	697.7508	558.4109	521.3005	Group2
8h-1	230.7888	149.0967	285.5434	248.079	Group2

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8h-2	222.345	131.3232	277.1254	242.1758	Group3
8h-3	273.8767	167.6216	320.4036	286.5982	Group3
12h-1	239.249	141.7303	279.4635	232.1544	Group3
12h-2	252.1497	147.359	288.996	243.3182	Group3
16h-1	226.2616	146.0414	284.8936	258.7989	Group3
16h-2	214.081	138.1432	277.3383	243.8137	Group3
16h-3	299.1992	209.131	342.5529	324.0587	Group3
24h-1	334.845	226.4543	369.0168	407.3281	Group4
24h-2	347.7744	261.7876	379.8865	406.6919	Group4
24h-3	315.3767	225.3518	365.1568	383.219	Group4
32h-1	286.9952	195.4727	332.1823	371.5529	Group4
32h-2	299.0332	218.8288	343.1931	380.1826	Group4
32h-3	261.7968	184.9235	319.7706	334.5194	Group4
40h-1	262.8648	167.8326	324.1094	366.8979	Group4
40h-2	257.8077	169.8686	316.6211	362.3634	Group4
40h-3	254.5887	184.1729	319.7806	350.5959	Group4
48h-1	262.8003	143.5924	312.7345	351.9222	Group4
48h-2	253.5572	148.9475	305.4050	346.3333	Group4
48h-3	338.3254	267.6897	379.5001	421.7295	Group4

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Note: Group1 denotes samples of 0 h (R-PMR), Group2denotes samples of 4 h, Group3 denotes samples of 8 to 16 h and Group4 denotes samples of 24 to 48 h

Table.5 The prediction of 35 sample depending on the 4 different discriminant functions scores

Sample name	Discriminant functions score				Prediction
	Group1	Group2	Group3	Group4	
S1	495.1874	396.0188	443.9055	404.5508	Group1
S2	569.9564	475.8026	503.4594	467.9002	Group1
S3	481.3273	394.9448	440.2833	405.2898	Group1
S4	451.3689	343.7183	429.8273	398.7956	Group1
S5	542.5472	466.388	490.1531	470.6152	Group1

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S6	481.8308	391.022	434.2839	385.6863	Group1
S7	505.8627	397.4449	452.3316	421.6209	Group1
S8	552.9518	473.4268	499.565	468.7872	Group1
S9	562.4498	481.9678	497.7762	466.9084	Group1
S10	541.1245	439.22	481.157	458.5999	Group1
S11	519.2018	452.041	464.2325	427.2706	Group1
S12	483.5436	361.9699	446.5619	404.2643	Group1
Z1	-542.224	-697.332	-312.926	-440.369	Group3
Z2	-125.898	-302.42	32.95553	-36.362	Group3
Z3	568.6966	720.4801	538.6208	632.584	Group2
Z4	-123.735	-278.387	37.18831	-43.1512	Group3
Z5	488.1378	713.6765	492.5701	553.6499	Group2
Z6	-323.377	-546.953	-138.732	-250.873	Group3
Z7	-497.102	-787.988	-298.719	-496.738	Group3
Z8	93.13856	81.82741	173.2194	179.7277	Group4
Z9	-379.549	-578.719	-190.125	-394.103	Group3
Z10	149.3714	120.1374	231.8514	209.0509	Group3
Z11	295.1615	142.8508	324.7297	383.072	Group4
Z12	308.1999	322.2907	343.3908	391.0132	Group4

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Z13	214.7621	293.449	273.225	326.784	Group4
Z14	347.636	399.8933	369.4687	471.3786	Group4
Z15	38.19232	-2.53979	156.111	188.6083	Group4
Z16	-378.665	-491.944	-181.058	-275.225	Group3
Z17	-108.522	-249.099	22.99999	-8.14454	Group3
Z18	-123.489	-244.781	10.92012	-28.0452	Group3
Z19	118.5649	93.63506	187.6745	169.4295	Group3
Z20	167.3504	143.5854	236.1682	290.5901	Group4
Z21	385.5307	353.8848	401.8973	440.4527	Group4
Z22	183.3692	191.1503	249.4224	292.7976	Group4
Z23	122.3098	-158.092	187.556	160.7807	Group3

Note: S1-12 present R-PMR sample. Z1-22 2 present P-PMR sample. Group1 denotes samples of 0 h(R-PMR), Group2 denotes samples of 4 h, Group3 denotes samples of 8 to 16 h and Group4 denotes samplesR of 24 to 48 h

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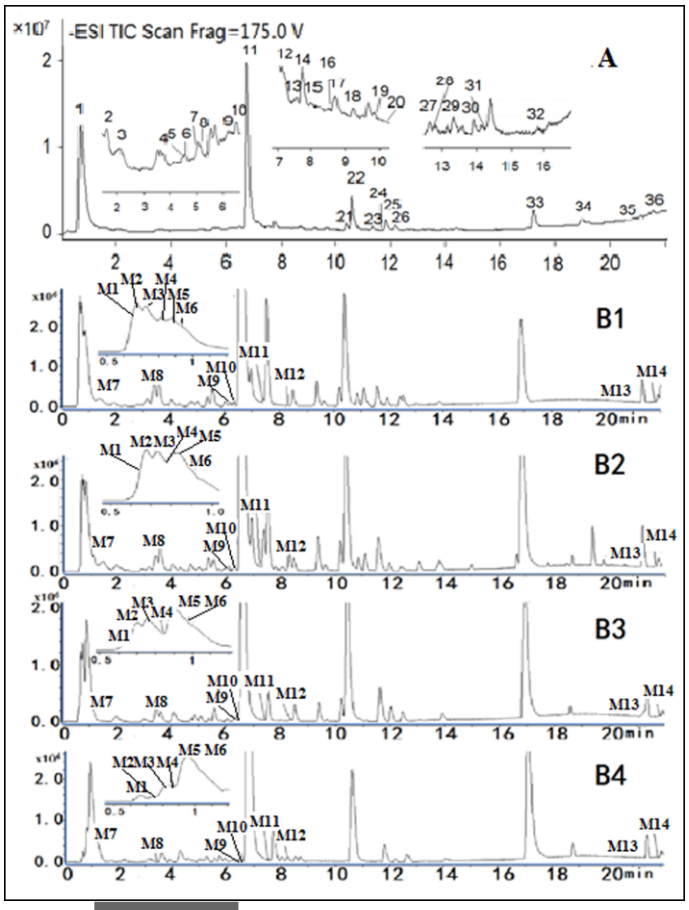

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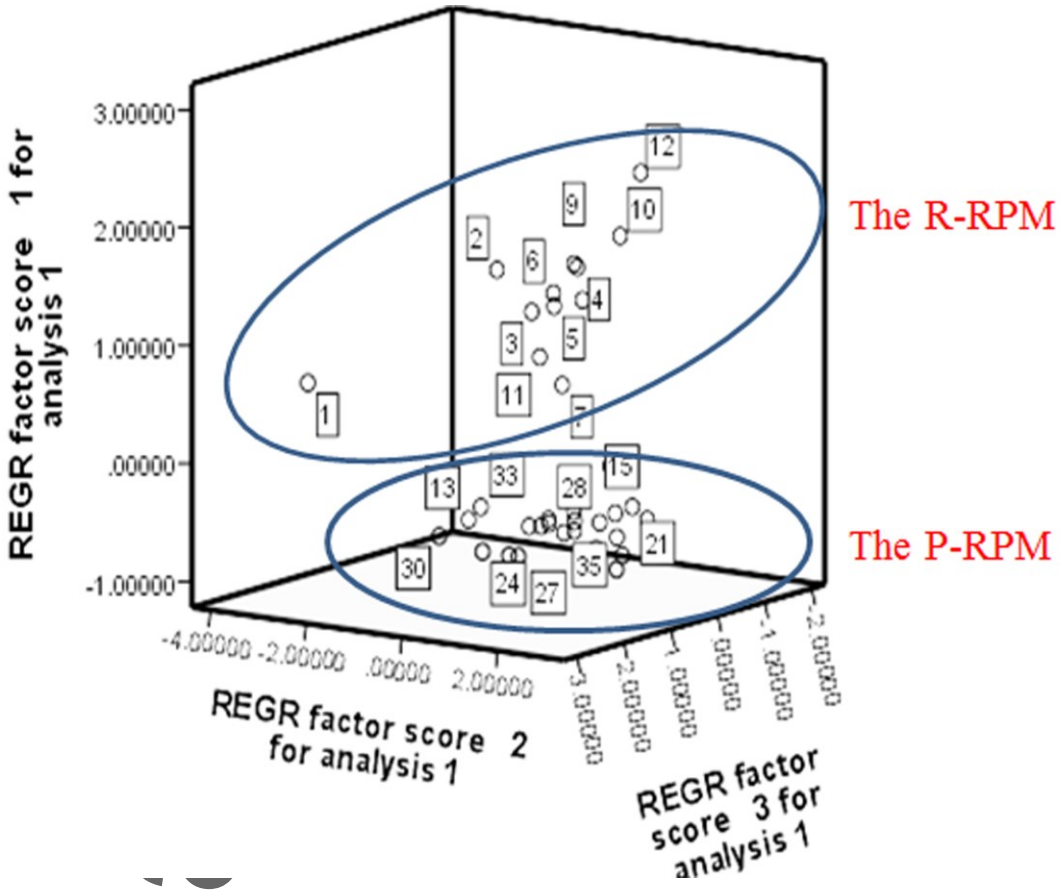

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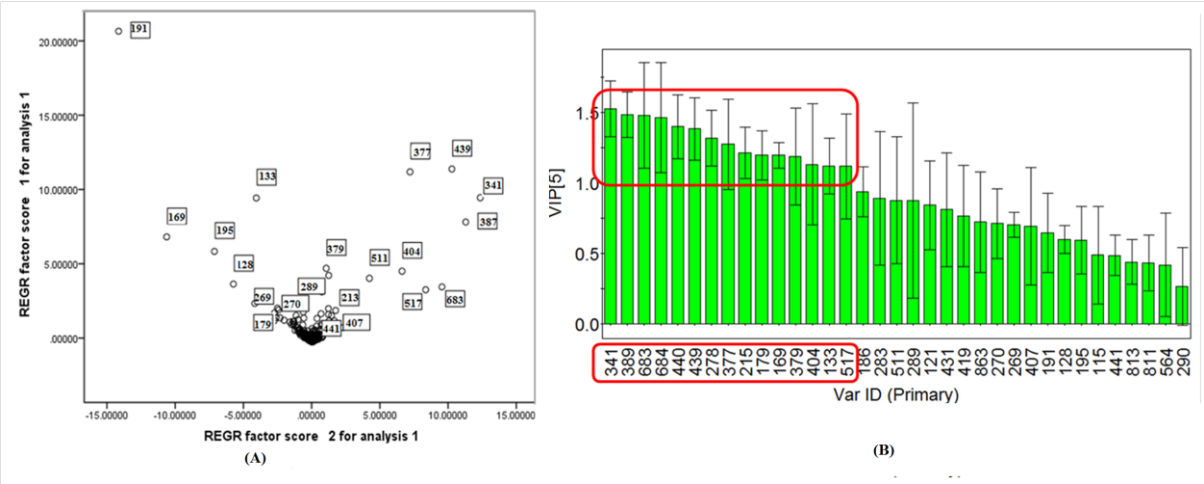

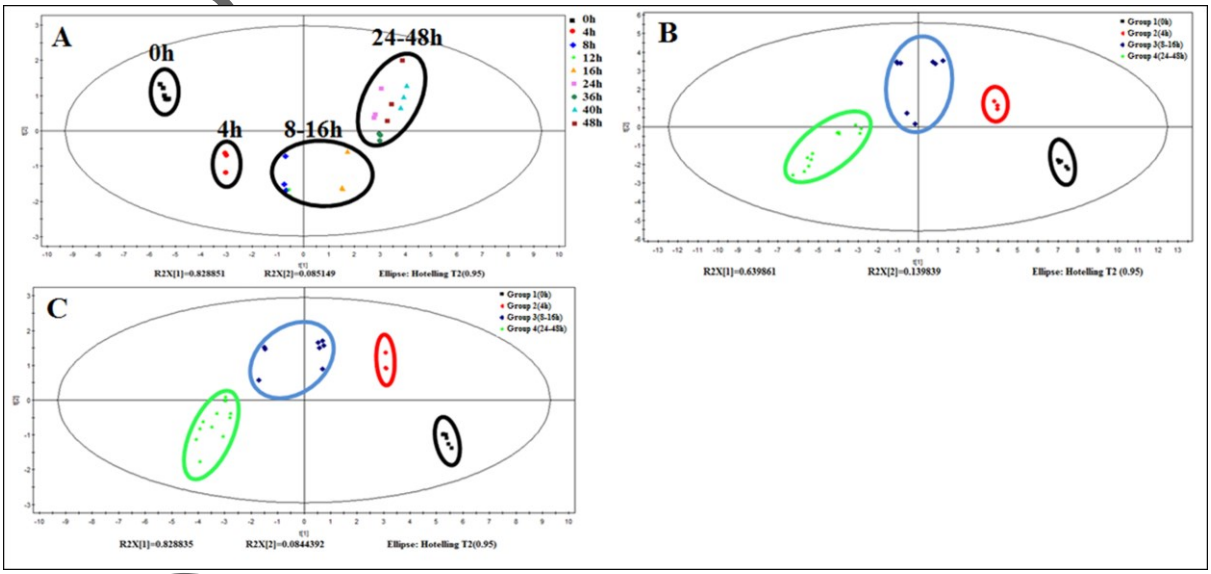

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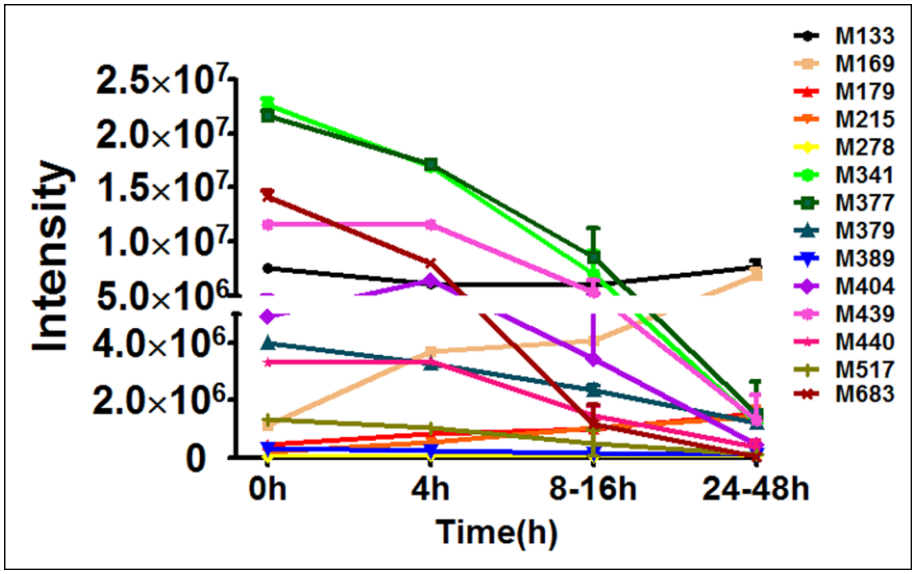

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