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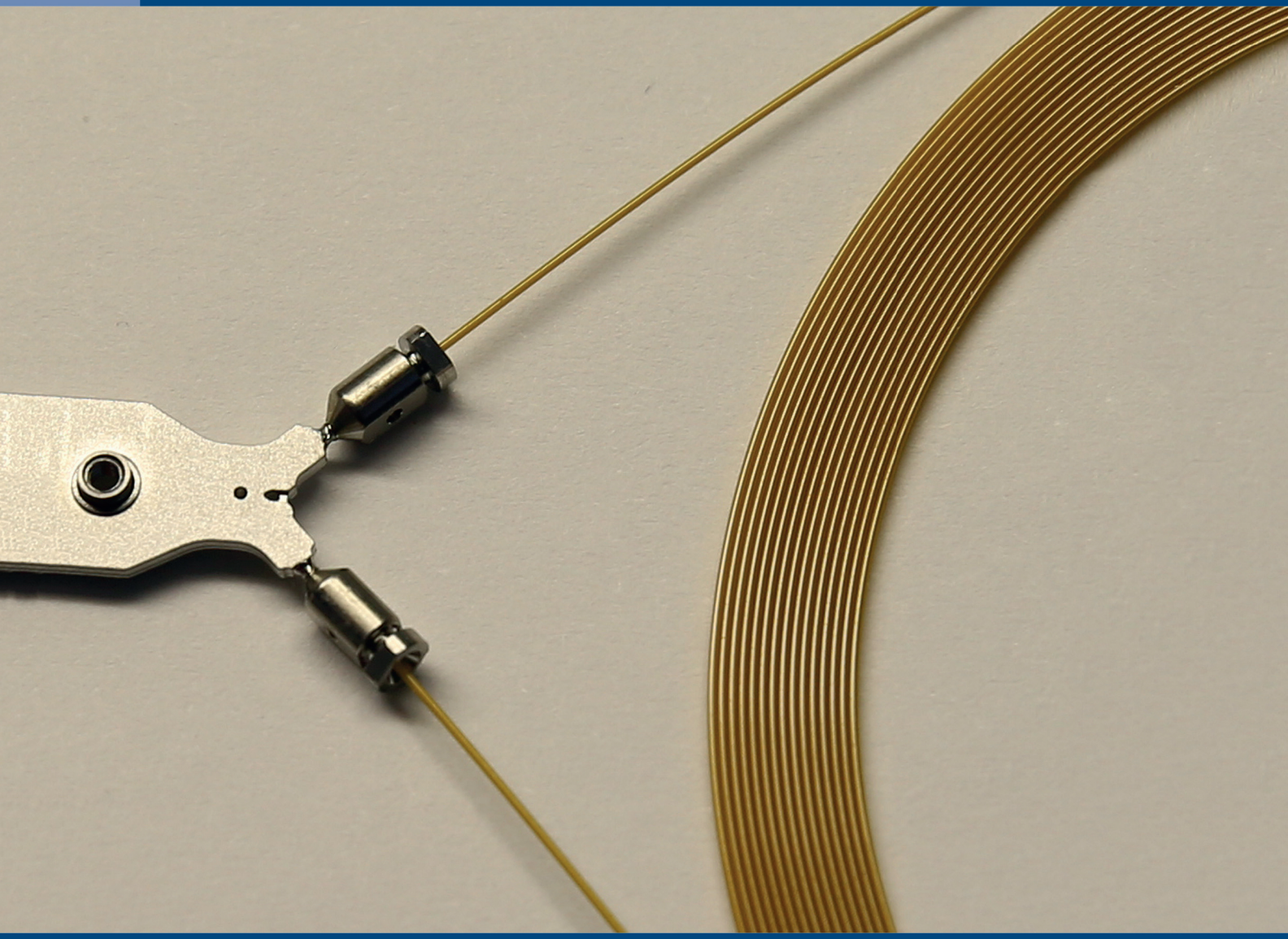

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RESEARCH ARTICLE

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# 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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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. Twelve 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*.

**KEYWORDS**

Fisher's discriminant analysis, metabolomics, *Polygoni Multiflora Radix*, principal component analysis, traditional Chinese medicine

## 1 | INTRODUCTION

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

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

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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 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, 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 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 preparation

#### 2.3.1 | Preparation of P-PMR extract

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 concentration of 1.0 mg/mL 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 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.

## 2.6 | Data analysis

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 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.

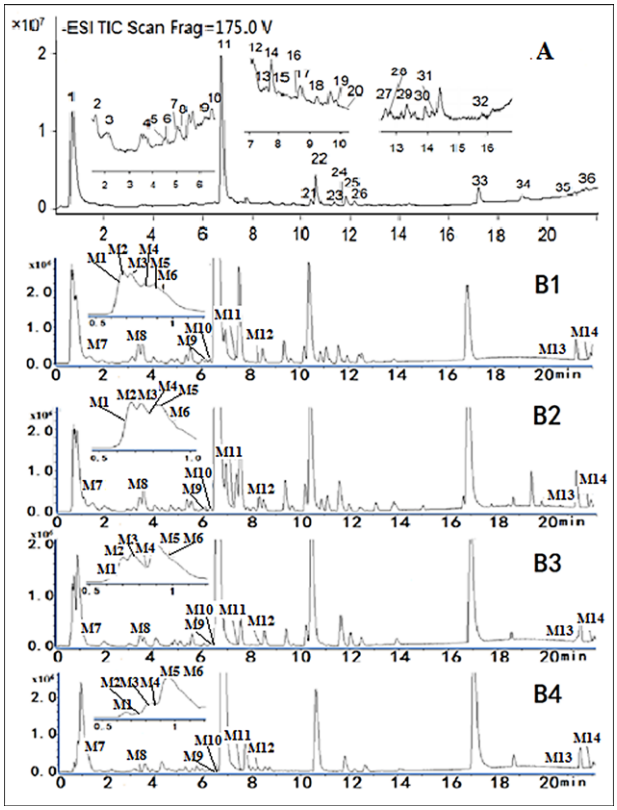


**FIGURE 1** The total ion chromatogram (TIC) of the main components in *Polygonum multiflorum* extract (A) and the representative chromatograms of the 4 groups. B1(R-PMR processed for 0 h), B2 (P-PMR processed for 4 h), B3(P-PMR processed for 8–12 h) and B4(P-PMR processed for 16–32 h).(M1-M14 present M683, M684, M377, M379, M404, M133, M169, M179, M389, M439, M440, M341, M278 and M215)

# 3 | RESULTS

## 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 is 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. A representative chromatogram of PMR is shown in Fig. 1A.

**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 |
| Physcion-8-*O*-glucoside | 2.40 | 2.12 | 2.14 |
| Rhein | 1.83 | 2.49 | 2.68 |
| Emodin | 1.77 | 1.49 | 2.86 |

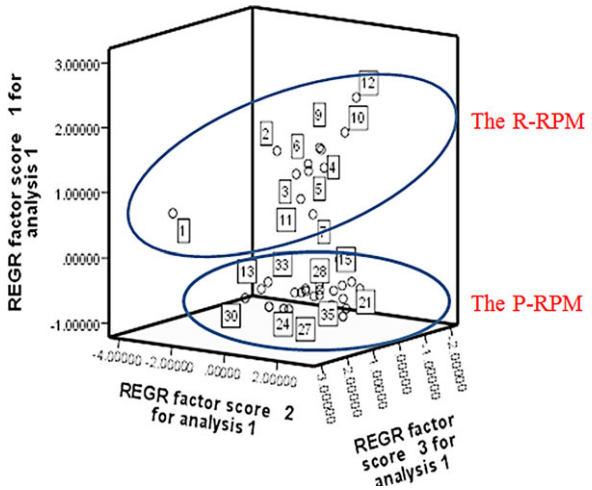


**FIGURE 2** The score plot of the PCA of samples based on the 1256 variables with $p < 0.05$

## 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 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 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.

## 3.4 | 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

### 3.4.1 | 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

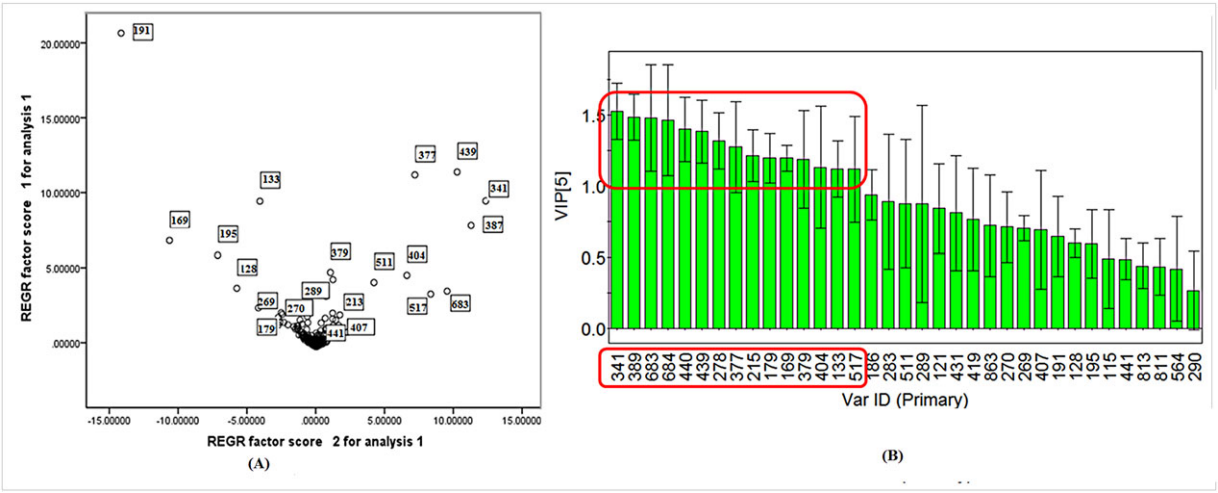


**FIGURE 3** The results of screened marker metabolites and the numbers present the molecular weight of metabolites and one circle presents a metabolite and the results of screened marker metabolites and the selected 15 marker metabolites according to VIP value (>1)

abundant ions, which 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 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].

### 3.4.2 | 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 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 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 it with the reference standard.

### 3.4.3 | 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*

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

| No. | | [MS⁻] | MS/MS | ppm | Formula | Compound |
|---|---|---|---|---|---|---|
| 1 | 0.786 | 403.1056 | 241.1022, 213.1123, 223.0221, 195.0324, 169.1231 | 0.21 | $C_{20}H_{20}O_9$ | Tetrahydroxy-phenanthrene-*O*-hexoside |
| 2 | 1.409 | 169.0141 | | 6.6 | $C_7H_6O_5$ | Gallic acid |
| 3 | 2.116 | 419.1653 | 213.1233, 195.1119, 128.0349, 101.0712 | 1.5 | $C_{20}H_{21}O_8$ | Rhaponticoside |
| 4 | 3.725 | 289.0724 | 215.0714, 173.0535, 149.0206, 125.0215, 109.0283 | 2.2 | $C_{15}H_{14}O_6$ | Catechin |
| 5 | 4.48 | 289.0724 | 276.4677, 205.0499, 163.0368, 131.0053, 109.0317 | 2.2 | $C_{15}H_{14}O_6$ | Epicatechin |
| 6 | 4.542 | 393.1603 | 231.1265, 187.1096, 189.0750 | 1.94 | $C_{19}H_{20}O_9$ | Hydroxymusizin-*O*-hexoside |
| 7 | 4.963 | 269.0120 | 240.0555, 211.1021 | 3.89 | $C_{15}H_{10}O_5$ | Aloe-emodin |
| 8 | 5.081 | 531.1498 | 487.0979, 283.0637, 239.0802 | 1.88 | $C_{26}H_{28}O_{12}$ | Physcion-8-*O*-(6′-*O*-malonyl)-glucoside |
| 9 | 6.227 | 567.1719 | 405.1198, 243.0657, 215.1021, 149.0245 | 0.05 | $C_{26}H_{32}O_{14}$ | Tetrahydroxystilbene-*O*-di-hexoside |
| 10 | 6.484 | 389.1168 | | 3.89 | $C_{20}H_{22}O_8$ | Polygonin |
| 11 | 6.748 | 811.0644 | 405.1180, 243.0648, 225.3022, 215.1024, 149.2312, 137.0237 | 1.03 | $C_{20}H_{22}O_9$ | 2,3,5,4′-Tetrahydroxystil-bene-2-*O*-β-D-glucoside |
| 12 | 7.145 | 407.1184 | 245.0656, 230.0237, 215.1011 | 0.95 | $C_{20}H_{24}O_9$ | Torachrysone-8-*O*-glucoside |
| 13 | 7.574 | 441.0853 | 331.1021, 289.0023, 169.3201 | 2.31 | $C_{22}H_{18}O_{10}$ | Catechingallate |
| 14 | 7.728 | 557.1162 | 405.1142, 313.0542, 243.0645, 169.0130 | 2.49 | $C_{27}H_{26}O_{13}$ | Tetrahydroxystilbene-*O*-(galloyl)-hexoside |
| 15 | 7.970 | 557.1034 | 405.1172, 313.0550, 243.0646, 169.0125 | 2.49 | $C_{27}H_{26}O_{13}$ | Tetrahydroxystilbene-*O*-(galloyl)-hexoside |
| 16 | 8.433 | 121.0295 | 92.0254, 65.0383 | 2.06 | $C_7H_6O_2$ | *p*-Hydroxybenzaldehyde |
| 17 | 8.681 | 421.1146/ 467.0823 | 259.0563 | 1.43 | $C_{20}H_{22}O_{10}$ | Pentahydroxystilbene-*O*-hexoside |
| 18 | 9.242 | 227.2002 | 202.0698, 176.0559, 99.9223, 91.0179, 73.5614 | 3.35 | $C_{14}H_{12}O_3$ | Resveratrol |
| 19 | 9.865 | 581.1636 | 387.1131, 331.0592, 243.0638, 405.0387 | 4.9 | $C_{30}H_{30}O_{12}$ | Tetrahydroxystilbene-2-*O*-(feruloyl)-hexoside |
| 20 | 10.202 | 551.1551 | 405.1134, 307.0819, 243.0658 | 0.57 | $C_{29}H_{28}O_{11}$ | Tetrahydroxystilbene-2-*O*-(coumaroyl)-hexoside |
| 21 | 10.472 | 407.1336 | 245.0800, 230.0568, 215.0348 | 0.12 | $C_{20}H_{24}O_9$ | Torachrysone-8-*O*-β-D-glucoside |
| 22 | 10.597 | 863.2053/ 431.1386 | 269.0448, 225.0540 | 0.86 | $C_{21}H_{20}O_{10}$ | Emodin-8-*O*-glucoside |

(Continues)

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 $[M–H]^-$

**TABLE 2** (Continued)

| No. | | [MS⁻] | MS/MS | ppm | Formula | Compound |
|---|---|---|---|---|---|---|
| 23 | 11.324 | 517.1009 | 473.1057, 269.0443 | 0.44 | $C_{24}H_{22}O_{13}$ | Emodin-*O*-(malonyl)-hexoside |
| 24 | 11.634 | 285.0380 | 257.0407, 241.0483, 211.0384, 268.0361 | 0.7 | $C_{25}H_{10}O_6$ | Citreorosein |
| 25 | 11.786 | 445.0595 | 283.0595, 240.0415 | 0.76 | $C_{21}H_{20}O_{10}$ | Physcion-8-*O*-glucoside |
| 26 | 12.241 | 473.1089 | 377.0121, 269.0453 | 0.48 | $C_{23}H_{22}O_{11}$ | Aloe-emodin-8-*O*-(6'-*O*-acetyl)-glucoside |
| 27 | 12.596 | 329.2322 | 229.1418, 211.13, 183.1392, 99.0808, 57.0335 | 1.44 | $C_{17}H_{14}O_7$ | Aurantio-obtusin |
| 28 | 12.794 | 473.1124 | 311.1052, 269.0477, 207.8834, 102.9316 | 1.02 | $C_{23}H_{22}O_{11}$ | Acetyl emodin-*O*-hexoside |
| 29 | 13.339 | 865.2084 | 577.0965, 289.0433 | 1.48 | $C_{42}H_{40}O_{20}$ | Trimer of catechin |
| 30 | 13.934 | 865.2053 | 577.0925, 289.1403 | 1.48 | $C_{42}H_{40}O_{20}$ | Prodelphinidin |
| 31 | 14.127 | 283.0606 | 240.0409 | 3.76 | $C_{15}H_8O_6$ | Rhein |
| 32 | 15.879 | 447.1321 | 243.9898 | 4.4 | $C_{22}H_{23}O_{10}$ | Tetrahydroxystilbene-*O*-(acetyl)-hexoside |
| 33 | 17.188 | 269.0460 | 241.0491, 225.0549, 210.0312, 197.0598, 181.0122 | 3.89 | $C_{15}H_{10}O_5$ | Emodin |
| 34 | 18.939 | 283.0399 | 268.1120, 240.0482, 212.1121, 184, 0213 | 0.88 | $C_{16}H_{12}O_5$ | Physcion |
| 35 | 20.596 | 577.1357 | 425.0873, 289.1143, 287.0559 | 1.82 | $C_{30}H_{26}O_{12}$ | Procyanidin B |
| 36 | 21.417 | 253.2173 | 225.0423, 210.0111, 181.1011 | 1.9 | $C_{15}H_{10}O_4$ | Chrysophanol |

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**), 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].

### 3.4.4 | Identification of naphthalenes

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].

### 3.4.5 | 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 is listed in Table 2.

### 3.5 | 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, 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 are listed in Table 3. Among the 35 maker metabolites, 15 compounds were identified according to our results of qualitative analysis. The other 20 compounds are still unknown and our identification work will continue.

### 3.6 | 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 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 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 markers 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:

$$\begin{aligned}\text{Group1} = {} & 0.000065885295x1 + 0.000086776115x2 \\ & -0.000000756662x3 - 0.000157974117x4 \\ & -0.001301271997x5 + 0.000046073611x6 \\ & -0.000024018062x7 + 0.000042095163x8 \\ & -0.000673736933x9 + 0.000004711657x10 \\ & -0.000042310637x11 + 0.000283186084x12 \\ & +0.000006733512x13 - 0.000029951111x14 \\ & -518.485421295047\end{aligned}$$

$$\begin{aligned}\text{Group2} = {} & 0.00004582315x1 + 0.000132116003x2 \\ & -0.00014813681x3 - 0.000157679338x4 \\ & +0.000624415946x5 + 0.000071567204x6 \\ & -0.000039769481x7 + 0.000100790637x8 \\ & -0.001579335678x9 + 0.000002449869x103 \\ & -0.000046686166x11 + 0.000337252255x12 \\ & +0.000002219651x13 - 0.000036448989x14 \\ & -696.409882440827\end{aligned}$$

$$\begin{aligned}\text{Group3} = {} & 0.000050911879x1 + 0.000064893671x2 \\ & -0.000026210477x3 - 0.000101949668x4 \\ & -0.000689318802x5 + 0.000033893287x6 \\ & -0.000021532886x7 + 0.000041543332x8 \\ & -0.000488599467x9 + 0.000003184608x10 \\ & -0.000034489071x11 + 0.000230166438x12 \\ & +0.000004439329x13 - 0.000022758896x14 \\ & -297.312167291817\end{aligned}$$

$$\begin{aligned}\text{Group4} = {} & 0.000068735928x1 + 0.00008846915x2 \\ & -0.000048268874x3 - 0.00011361905x4 \\ & -0.001528276717x5 + 0.000027687492x6 \\ & -0.000014014032x7 + 0.000018717914x8 \\ & -0.00085575779x9 + 0.000004890566x10 \\ & -0.000031915674x11 + 0.00023368023x12 \\ & +0.000008409557x13 - 0.000018287617x14 \\ & -376.383930338424\end{aligned}$$

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, 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.

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

| No. | Marker | Rt. | MS | MS/MS | ppm | Formula | Compound |
|---|---|---|---|---|---|---|---|
| 1 | M115 | 0.601 | 115.0022 | | | | Unknown |
| 2 | M683 | 0.669 | 683.2240 | | | | Unknown |
| 3 | M684 | 0.669 | 684.2269 | | | | Unknown |
| 4 | M377 | 0.702 | 377.0871 | 179.0354, 119.0345, 101.0233, 89.0248, 71.0136, 59.0146 | | | Unknown |
| 5 | M379 | 0.702 | 379.0831 | 179.0354, 119.0345, 101.0233, 89.0248, 71.0136, 59.0146 | | | Unknown |
| 6 | M404 | 0.786 | 404.1061 | 61.9891 | | | Unknown |
| 7 | M133 | 0.801 | 133.0145 | | | | Unknown |
| 8 | M191 | 0.920 | 191.0205 | 57.0357, 85.0304, 87.0089, 111.0076 | | | Unknown |
| 9 | M169 | 1.409 | 169.0141 | | 6.6 | $C_7H_6O_5$ | Gallic acid |
| 10 | M128 | 1.796 | 128.0354 | 109.6663, 103.2380, 67.9247, 60.9239 | | | Unknown |
| 11 | M419 | 2.205 | 419.1682 | | | | Unknown |
| 12 | M186 | 3.464 | 186.0555 | 142.0656, 116.0488 | | | Unknown |
| 13 | M179 | 3.543 | 179.0549 | | | | Unknown |
| 14 | M289 | 3.725 | 289.0724 | 215.0714, 173.0535, 149.0206, 125.0215, 109.0283 | 2.2 | $C_{15}H_{14}O_6$ | Catechin |
| 15 | M289 | 4.48 | 289.0724 | 276.4677, 205.0499, 163.0368, 131.0053, 109.0317 | 2.2 | $C_{15}H_{14}O_6$ | Epicatechin |
| 16 | M195 | 5.724 | 195.0508 | | | | Unknown |
| 17 | M389 | 6.484 | 389.1168 | | 3.89 | $C_{20}H_{22}O_8$ | Polygonin |
| 18 | M439 | 6.748 | 439.0816 | | | | Unknown |
| 19 | M811 | 6.748 | 811.0644 | 405.1180, 243.0648, 225.3022, 215.1024, 149.2312, 137.0237 | 1.03 | $C_{20}H_{22}O_9$ | 2,3,5,4′-Tetrahydroxystilbene-2-*O*-β-D-glucoside |
| 20 | M813 | 6.748 | 813.0644 | 405.1180, 243.0648, 225.3022, 215.1024, 149.2312, 137.0237 | 1.03 | $C_{20}H_{22}O_9$ | 2,3,5,4′-Tetrahydroxystilbene-2-*O*-β-D-glucoside |
| 21 | M407 | 7.145 | 407.1184 | 245.0656, 230.0237, 215.1011 | 0.95 | $C_{20}H_{24}O_9$ | Torachrysone-8-*O*-glucoside |
| 22 | M440 | 7.574 | 441.0853 | 331.1021, 289.0023, 169.3201 | 2.31 | $C_{22}H_{18}O_{10}$ | Catechingallate |
| 23 | M441 | 7.574 | 441.0853 | 331.1021, 289.0023, 169.3201 | 2.31 | $C_{22}H_{18}O_{10}$ | Catechingallate |
| 24 | M121 | 8.433 | 121.0295 | 92.0254, 65.0383 | 2.06 | $C_7H_6O_2$ | *p*-Hydroxybenzaldehyde |
| 25 | M341 | 8.714 | 341.1097 | 249.0698, 89.0241, 59.0141 | | | Unknown |
| 26 | M511 | 9.655 | 511.0576 | 431.0976, 121.0283 | | | Unknown |

(Continues)

**TABLE 3** (Continued)

| No. | Marker | Rt. | MS | MS/MS | ppm | Formula | Compound |
|---|---|---|---|---|---|---|---|
| 27 | M431 | 10.597 | 431.1386 | 269.0448, 225.0540 | 0.86 | $C_{21}H_{20}O_{10}$ | Emodin-8-*O*-glucoside |
| 28 | M517 | 11.324 | 517.1009 | 473.1057, 269.0443 | 0.44 | $C_{24}H_{23}O_{13}$ | Emodin-8-*O*-(6′-*O*-malonyl)-glucoside |
| 29 | M865 | 13.934 | 865.2053 | 577.0925, 289.1403 | 1.48 | $C_{42}H_{40}O_{20}$ | Prodelphinidin |
| 30 | M269 | 17.188 | 269.0460 | 241.0491, 225.0549, 210.0312, 197.0598, 181.0122 | 3.89 | $C_{15}H_{10}O_5$ | Emodin |
| 31 | M270 | 17.188 | 270.0496 | 241.0491, 225.0549, 210.0312, 197.0598, 181.1021 | 3.89 | $C_{15}H_{10}O_5$ | Emodin |
| 32 | M564 | 18.708 | 564.3323 | | | | Unknown |
| 33 | M283 | 18.939 | 283.0399 | 268.1120, 240.0482, 212.1121, 184, 0213 | 0.88 | $C_{16}H_{12}O_5$ | Physcion |
| 34 | M278 | 20.872 | 278.0894 | | | | Unknown |
| 35 | M215 | 22.489 | 215.0327 | | | | Unknown |

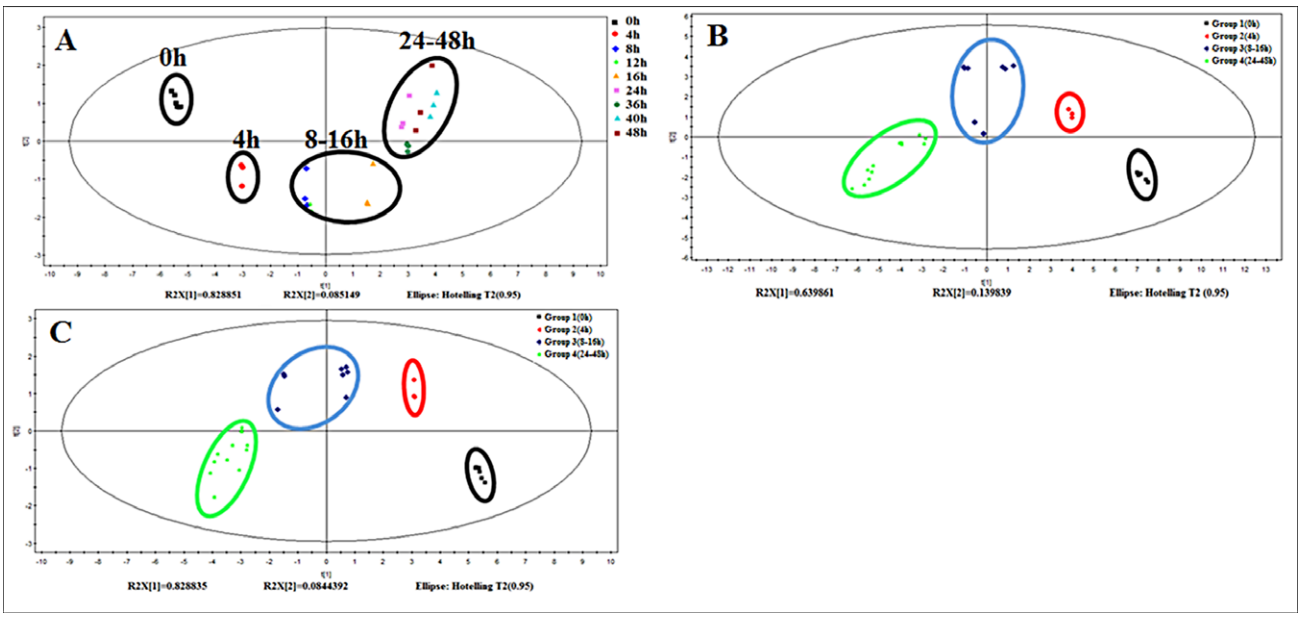


**FIGURE 4** PCA model for known 29 samples at different processing times (0, 4, 8, 12, 16, 24, 32, 40, and 48 h) and prediction for clustering another four groups (A); the PLS-DA model for 29 samples of four different processing times depending on 35 maker metabolites (B); the PLS-DA model for 29 samples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

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 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.

**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 | Group 1 |
| 0h–2 | 535.9555 | 450.1285 | 482.2046 | 450.8902 | Group 1 |
| 0h–3 | 523.0573 | 441.1327 | 471.8197 | 439.5416 | Group 1 |
| 0h–4 | 506.8386 | 416.3425 | 461.1772 | 428.2384 | Group 1 |
| 0h–5 | 506.4268 | 420.5351 | 459.7598 | 425.3151 | Group 1 |
| 0h–6 | 497.6219 | 410.4663 | 454.6204 | 417.8401 | Group 1 |
| 4h–1 | 620.1771 | 724.5048 | 568.5795 | 537.1318 | Group 2 |
| 4h–2 | 576.3855 | 658.6562 | 531.7202 | 500.3762 | Group 2 |
| 4h–3 | 613.4444 | 697.7508 | 558.4109 | 521.3005 | Group 2 |
| 8h–1 | 230.7888 | 149.0967 | 285.5434 | 248.079 | Group 2 |
| 8h–2 | 222.345 | 131.3232 | 277.1254 | 242.1758 | Group 3 |
| 8h–3 | 273.8767 | 167.6216 | 320.4036 | 286.5982 | Group 3 |
| 12h–1 | 239.249 | 141.7303 | 279.4635 | 232.1544 | Group 3 |
| 12h–2 | 252.1497 | 147.359 | 288.996 | 243.3182 | Group 3 |
| 16h–1 | 226.2616 | 146.0414 | 284.8936 | 258.7989 | Group 3 |
| 16h–2 | 214.081 | 138.1432 | 277.3383 | 243.8137 | Group 3 |
| 16h–3 | 299.1992 | 209.131 | 342.5529 | 324.0587 | Group 3 |
| 24h–1 | 334.845 | 226.4543 | 369.0168 | 407.3281 | Group 4 |
| 24h–2 | 347.7744 | 261.7876 | 379.8865 | 406.6919 | Group 4 |
| 24h–3 | 315.3767 | 225.3518 | 365.1568 | 383.219 | Group 4 |
| 32h–1 | 286.9952 | 195.4727 | 332.1823 | 371.5529 | Group 4 |
| 32h–2 | 299.0332 | 218.8288 | 343.1931 | 380.1826 | Group 4 |
| 32h–3 | 261.7968 | 184.9235 | 319.7706 | 334.5194 | Group 4 |
| 40h–1 | 262.8648 | 167.8326 | 324.1094 | 366.8979 | Group 4 |
| 40h–2 | 257.8077 | 169.8686 | 316.6211 | 362.3634 | Group 4 |
| 40h–3 | 254.5887 | 184.1729 | 319.7806 | 350.5959 | Group 4 |
| 48h–1 | 262.8003 | 143.5924 | 312.7345 | 351.9222 | Group 4 |
| 48h–2 | 253.5572 | 148.9475 | 305.4050 | 346.3333 | Group 4 |
| 48h–3 | 338.3254 | 267.6897 | 379.5001 | 421.7295 | Group 4 |

*Note*: Group 1 denotes samples of 0 h (R-PMR), Group2denotes samples of 4 h, Group 3 denotes samples of 8 to 16 h and Group 4 denotes samples of 24 to 48 h

To show the regular change of P-PMR samples with difference in processing time, it was essential to compare the content of screening 14 markers in different groups. Figure 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 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

**TABLE 5** The prediction of 35 sample depending on the four different discriminant functions scores

| | Discriminant functions score | | | | |
|---|---|---|---|---|---|
| **Sample name** | **Group 1** | **Group 2** | **Group 3** | **Group 4** | **Prediction** |
| S1 | 495.1874 | 396.0188 | 443.9055 | 404.5508 | Group 1 |
| S2 | 569.9564 | 475.8026 | 503.4594 | 467.9002 | Group 1 |
| S3 | 481.3273 | 394.9448 | 440.2833 | 405.2898 | Group 1 |
| S4 | 451.3689 | 343.7183 | 429.8273 | 398.7956 | Group 1 |
| S5 | 542.5472 | 466.388 | 490.1531 | 470.6152 | Group 1 |
| S6 | 481.8308 | 391.022 | 434.2839 | 385.6863 | Group 1 |
| S7 | 505.8627 | 397.4449 | 452.3316 | 421.6209 | Group 1 |
| S8 | 552.9518 | 473.4268 | 499.565 | 468.7872 | Group 1 |
| S9 | 562.4498 | 481.9678 | 497.7762 | 466.9084 | Group 1 |
| S10 | 541.1245 | 439.22 | 481.157 | 458.5999 | Group 1 |
| S11 | 519.2018 | 452.041 | 464.2325 | 427.2706 | Group 1 |
| S12 | 483.5436 | 361.9699 | 446.5619 | 404.2643 | Group 1 |
| Z1 | − 542.224 | − 697.332 | − 312.926 | − 440.369 | Group 3 |
| Z2 | − 125.898 | − 302.42 | 32.95553 | − 36.362 | Group 3 |
| Z3 | 568.6966 | 720.4801 | 538.6208 | 632.584 | Group 2 |
| Z4 | − 123.735 | − 278.387 | 37.18831 | − 43.1512 | Group 3 |
| Z5 | 488.1378 | 713.6765 | 492.5701 | 553.6499 | Group 2 |
| Z6 | − 323.377 | − 546.953 | − 138.732 | − 250.873 | Group 3 |
| Z7 | − 497.102 | − 787.988 | − 298.719 | − 496.738 | Group 3 |
| Z8 | 93.13856 | 81.82741 | 173.2194 | 179.7277 | Group 4 |
| Z9 | − 379.549 | − 578.719 | − 190.125 | − 394.103 | Group 3 |
| Z10 | 149.3714 | 120.1374 | 231.8514 | 209.0509 | Group 3 |
| Z11 | 295.1615 | 142.8508 | 324.7297 | 383.072 | Group 4 |
| Z12 | 308.1999 | 322.2907 | 343.3908 | 391.0132 | Group 4 |
| Z13 | 214.7621 | 293.449 | 273.225 | 326.784 | Group 4 |
| Z14 | 347.636 | 399.8933 | 369.4687 | 471.3786 | Group 4 |
| Z15 | 38.19232 | − 2.53979 | 156.111 | 188.6083 | Group 4 |
| Z16 | − 378.665 | − 491.944 | − 181.058 | − 275.225 | Group 3 |
| Z17 | − 108.522 | − 249.099 | 22.99999 | − 8.14454 | Group 3 |
| Z18 | − 123.489 | − 244.781 | 10.92012 | − 28.0452 | Group 3 |
| Z19 | 118.5649 | 93.63506 | 187.6745 | 169.4295 | Group 3 |
| Z20 | 167.3504 | 143.5854 | 236.1682 | 290.5901 | Group 4 |
| Z21 | 385.5307 | 353.8848 | 401.8973 | 440.4527 | Group 4 |
| Z22 | 183.3692 | 191.1503 | 249.4224 | 292.7976 | Group 4 |
| Z23 | 122.3098 | − 158.092 | 187.556 | 160.7807 | Group 3 |

*Note*: S1–12 present R-PMR sample. Z1–22 2 present P-PMR sample. Group 1 denotes samples of 0 h (R-PMR), Group 2 denotes samples of 4 h, Group 3 denotes samples of 8 to 16 h and Group 4 denotes samples R of 24 to 48 h.

available in the 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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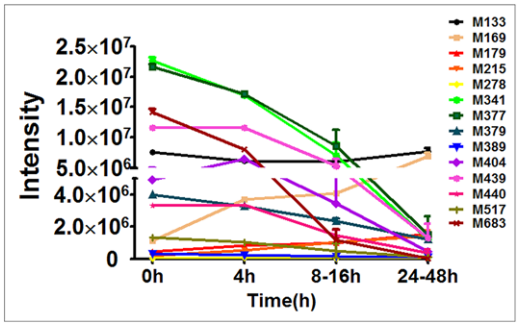


**FIGURE 5** The average intensity of 14 markers in the four different groups

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