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ORIGINAL ARTICLE

Integrating Background Subtraction and Metabolomics Technology to Study the Difference of Serum Chemical Profile Before and After Compatibility of *Radix Bupleuri* and *Radix Paeoniae Alba*

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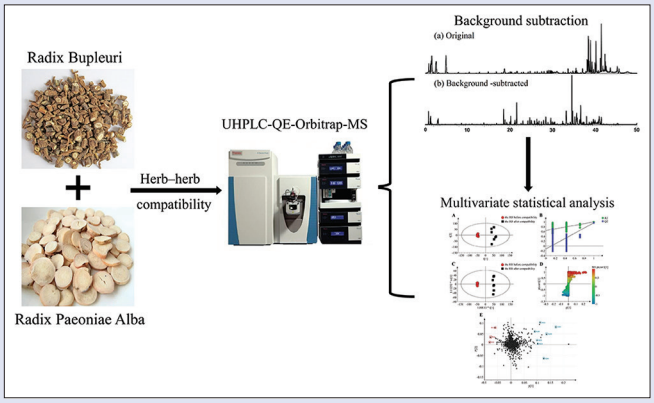
ABSTRACT

Background: *Radix Bupleuri-Radix Paeoniae Alba* (CH-BS) is a common clinical herb pair, which is often used to treat depression. However, there is little known about the difference in serum chemical profile before and after the compatibility of *Radix Bupleuri (*CH) and *Radix Paeoniae Alba* (BS). **Objectives:** Here, an effective strategy has been developed for screening differences of constituents *in vivo* before and after compatibility by integrating background subtraction and metabolomics technology. **Materials and Methods:** First, to obtain the chemical composition from the CH *in vivo* before compatibility, the Liquid chromatography/Mass spectrometry (LC/MS) data of serum samples in the control group were subtracted from serum samples in the CH group based on the background subtraction method. Moreover, the chemical composition from the CH *in vivo* after compatibility was obtained by subtracting the LC/MS data of serum samples in the BS group from serum samples in the CH-BS compatibility group. Finally, the difference in the chemical composition from the CH *in vivo* before and after compatibility was screened and analyzed by metabolomics. Meanwhile, the difference in chemical composition from the BS *in vivo* before and after the compatibility was found according to the same thought with BS. **Results:** The results showed that three prototype components were significantly decreased and seven metabolites were significantly increased in CH after compatibility. And eight prototype components and six metabolites were significantly increased in BS after compatibility. **Conclusion:** An effective strategy has been developed for screening differences of constituents *in vivo* before and after compatibility by combining background subtraction and metabolomics.

Key words: Background subtraction, compatibility, metabolomics, *Radix Bupleuri-Radix Paeonia Alba*, UHPLC-QE-Orbitrap-MS

SUMMARY

- The serum chemical profile research on herb–herb interactions.
- The integration strategies of background subtraction and metabolomics technology.
- To reveal the potential theoretical basis for the compatibility of CH and BS.



Abbreviations used: *CH: Radix Bupleuri; BS: Radix Paeoniae Alba*; TCM: Traditional Chinese medicine; BPC: Base peak chromatograms; PCA: Principal components analysis; PLS-DA: Partial least-squares discriminant analysis; OPLS-DA: Partial least-squares discriminant analysis.

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INTRODUCTION

Herb pairs are not only the fundamental composition units of TCM formulae but are also the centralized representatives of TCM compatibility.[1] Extensive research has shown that herb pairs exhibited improved pharmacological activities than the individual herb, and the research of herb pairs has drawn more attention in the field of herb-herb interactions. When there is herb-herb compatibility, it can achieve satisfactory therapeutic efficacy by obtaining synergistic effects and minimizing possible adverse reactions.[2-4] Currently, serum chemical profile studies of herb-herb interactions have been proved useful to

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clarify the basic principles of TCM compatibility, because they can accurately reflect the changes in pharmacodynamic substances basis in the body.[5] Therefore, the study of chemical interactions was extremely significant in the exploration of the compatibility of TCM.

Radix Bupleuri (*Bupleurum chinense* DC., CH) is the root of the umbelliferous plant Bupleurum falcatum Linne, which has been used as a TCM for more than 2,000 years and was first published in "*Shennong's Classic of Materia Medica*." Modern pharmacological research has proved that CH has antipyretic, sedative, analgesic, anti-inflammatory, anti-bacterial, immune-enhancing, anti-depressant, and anti-tumur effects.[6] The saikosaponins A and saikosaponins D were the main anti-depressant active ingredients.[7] Radix Paeoniae Alba (*Paeonia lactiflora* Pall., BS), as the drug first appeared in the "*Prescriptions for Fifty-two Diseases*", is the roots of the Ranunculaceae plant *Paeonia lactiflora* Pall. Modern pharmacological research has proved that BS has a range of pharmacological activities, such as analgesia, sedation, antispasmodic, anti-inflammatory, anti-tumor, liver protection, and anti-depressant.[8] Paeoniflorin and albiflorin were the major bioactive components for their anti-depressant effects.[9] CH and BS were both famous traditional herbal medicines that were often used in combination to improve the therapeutic effect. The CH-BS herb pair were recorded as the principal drug in "Xiaoyaosan" and "Sinisan," which was playing anti-depressant effect by "soothing liver and resolving melancholia".[10] Recent studies have shown that their anti-depressant effects were significantly improved when CH and BS were used as a whole herb pair.[11] However, there is little known about the content variations of the bioactive components in CH and BS before and after compatibility. Furthermore, studying the difference in the serum chemical profile before and after compatibility has become a key to elucidating the potential mechanism of herbal compatibility, which will help to elucidate the mechanism of CH-BS compatibility.

Recently, the identification of serum pharmacochemistry of herbal formulations was limited by the low content of some ingredients in herbals. However, Ultra high performance liquid chromatography-Q Exactive-Orbitrap-Mass spectrometry (UHPLC-QE-Orbitrap-MS) has become a reliable technique for the identification of known and unknown chemical compositions from a complex matrix.[12,13] Besides, background subtraction is a process in mass spectrometry data analysis that extracts ions and performs subtraction from the background or the sample matrix.[14] In the study of the serum chemical profile of herbal medicine, background subtraction technology provided an effective method to reduce matrix ions and clear LC-MS data in biological samples and was used to screen the prototype constituents and metabolites of herbal medicine in complex biological matrices. Multivariate statistical analysis is a key technology in metabolomics research. It can not only analyze the differences of small molecular endogenous metabolites in the body but also is used as a valuable tool for analyzing the complex components and metabolites of herbal medicine.[15] In this experiment, the UHPLC-HRMS background subtraction method combined with the metabolomics technique was used to analyze the overall chemical profile of CH and BS in rat serum before and after combination, respectively. Meanwhile, through the multivariate statistical analysis, the chemical components with significant changes after compatibility were discovered, and the potential mechanism of CH and BS compatibility was revealed from the perspective of the change rule of the serum chemical profile.

MATERIALS AND METHODS

Instruments

Thermo-Fisher Dionex UltiMate 3000 UHPLC-Q Exactive Orbitrap-MS (Thermo Fisher, USA) and Xcalibur workstation; TGL-16 high-speed desktop refrigeration centrifuge (Hunan Xiangyi Centrifuge Instrument Co., Ltd.); Rotary Evaporator (German Aika Group); Freeze Dryer (Ningbo Xinzhi Biotechnology Co., Ltd.).

Chemicals and reagents

CH (batch number: 180801, Neimenggu Province) and BS (batch number: 181102, Anhui Province) were purchased from the Anguo Qiao Chinese herbal sliced medicine Co. Ltd and authenticated by Prof. Xue-Mei Qin (Shanxi University). Saikosaponin A, saikosaponin D, saikosaponin C, saikosaponin B_2, paeoniflorin, albiflorin, oxypaeoniflorin, and methyl gallate were all purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Chromatography-grade methanol, acetonitrile, and formic acid were obtained from Fisher (Fairlawn, NJ, United States).

Preparation of CH-BS herb pair and their respective drug powders

According to the previous research,[10] to obtain the extract of CH or BS, 225 g of CH or 225 g of BS were soaked in 70% ethanol (1800 mL) for 1.5 h, and then extracted two times, each time for 2.5 h. Then, the combined extracts were concentrated in vacuo to 50 mL. To obtain the mixed extracts (the extract of CH-BS), 225 g of CH and 225 g of BS were mixed with 3600 mL/70% ethanol, and then prepared the mixed extracts based on the same procedure described earlier.

Animals and drug administration

Healthy male SPF grade SD rats (180 ± 20 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (Beijing, China, No. SCXK2020-0132). All rats were adapted to the lab environment for 7 days (room temperature 24 ± 3°C, relative humidity 55 ± 5%, and 12 h light-dark cycle). All experimental procedures were carried out following the NIH Guide, and the experiments had also obtained approval from the Animal Ethics Committee of Shanxi University (SXULL2020018). After 1 week of adaptation, according to the baseline of body weight, 28 rats with similar indicators were divided into four groups randomly, with seven rats in each group, as follows: (1) control group; (2) CH group, oral administration of CH extract: 30 g-herb/kg; (3) BS group, oral administration of BS extract: 30 g-herb/kg; and (4) CH-BS group, oral administration of mixed extracts: 60 g-herb/kg. All drugs were administrated to rats at a volume of 10 ml/kg body weight, and the control group were given water.

Collection and preparation of serum samples

After 1 week of adaptation and fasted for 12 h, all rats were given the corresponding drugs two times a day for 3 days. The blood samples (0.6 mL) were collected from the ophthalmic venous plexus into 2 mL EP tubes after the last oral administration at 15, 30, 45, 60, and 180 min. After blood collection, the serum samples were obtained by centrifugation of blood at 3600 rpm for 10 min. Then, the serum samples from the same rat at various time points were combined and kept frozen at – 80°C until further use.

For UHPLC-MS analysis, a 2-mL serum sample was spiked with 6 mL chilled methanol, vortexed for 1 min, and centrifuged at 4°C/13,500 rpm for 15 min. Then, the supernatant was transferred and dried under N_2. The dry extract was reconstituted in 500 ul methanol, vortexed for 1 min, and centrifuged at 4°C/13,500 rpm for 15 min. Finally, the supernatants were collected for UHPLC-MS analysis.

UHPLC-MS/MS method

A Dionex UltiMate 3000 UHPLC system combined with a Q Exactive Orbitrap-MS spectrometer and an Xcalibur workstation was used to

acquire LC-MS/MS data. A UPLC T_3 column (2.1 mm × 100 mm, 1.8 μm) was used for chromatographic separation. The mobile phase system included water/0.1% formic acid (solvent A) and acetonitrile (solvent B) under a gradient elution as follows: 0-8 min, 5%-10% B; 8-18 min, 10%-17% B; 18-26 min, 17%-20% B; 26-36 min, 20%-60% B; 36-40 min, 60%-100% B; 40-43 min, 100%; 43-46 min, 100%-5% B; and 46-50 min, 5%B. The flow rate was set at 0.3 ml/min. The injection volume was 10 μL. All samples were analyzed under positive and negative ionization modes via heated electrospray ionization (HESI) source. The detailed parameters were as follows: spray voltage of 3.5 kV for the positive mode and 2.5 kV for the negative mode, the capillary temperature of 320°C, the sheath of 35 arbitrary units, and auxiliary gas flow rates of 10 arbitrary units. The range of mass scanning was set from 50 to 1000 (*m/z*).

Implementation of the background subtraction algorithm

The UHPLC-MS data of the analyte samples were processed using an in-house background subtraction algorithm Strip software.[14] In this study, for the chemical profile of the CH *in vivo* before compatibility, the control group rat's serum samples were used to provide background as control samples, and the CH group rat's serum samples were used as analyte samples. Using the background subtraction method, the LC/MS data of the control samples (the control group rat's serum samples) was subtracted from the analysis sample (the CH group rat's serum samples), in order to obtain the LC/MS data of CH before compatibility without matrix interference. Besides, for the chemical profile of the CH *in vivo* after the compatibility, the BS group rat's serum samples were used to provide background as control samples, and the CH-BS compatibility group rat's serum samples were used as analyte samples. Using the background subtraction method, the LC/MS data of the control samples (the BS group rat's serum samples) were subtracted from the analysis sample (the CH-BS compatibility group rat's serum samples) to obtain the LC/MS data of CH after compatibility. In the same way, the chemical profile of the BS *in vivo* before and after compatibility was obtained based on the same procedure described earlier. The processing parameters: scan time window was 1 min; mass error tolerance was 5 ppm; scaling factor was 15; mass window was 0.05 Da; and retention time window was 0.2.

UHPLC-MS/MS data processing

The UHPLC-MS/MS data raw files after background subtraction were imported into the Compound Discoverer 3.3 software for peak alignment and detection. The parameters were set as follows: mass range: 50-1000 Da; Mass Tolerance: 5 ppm; RT tolerance [min]: 0.05; and Signal-to-noise ratio threshold (S/N Threshold): 8. The retention time, molecular formula, precise molecular weight, and peak area information obtained from Compound Discoverer 3.3 were imported into Microsoft Office Excel 2019, and the peak areas were treated by area normalization.

Statistical analysis

The background-subtracted data were further imported into SIMCA-P 14.1 (Umetrics, Sweden) for multivariate statistical analyses. The most significant difference variable was screened by combining VIP (variable importance in the projection) >1 in the V-S-plot plot and $P < 0.05$ in the *t*-test of the independent sample. Statistical analysis was performed using SPSS software (version 19.0). All the data were expressed as mean and standard deviation (mean ± SD). Statistical differences between two groups were compared using Student's t-test, and the differences between multiple groups were compared by one-way ANOVA.

RESULTS

Analysis of prototype compounds in serum sample

UHPLC-Q-Exactive Orbitrap-MS combined with Xcalibur workstation was applied to identify the absorbed constituents and metabolites from the CH-BS herb pair. In this study, the peaks which appeared at the same position in the chromatograms of both the serum samples of the CH-BS group and the extracts but not in the chromatograms of serum samples of the control group were considered as absorbed prototype constituents. Finally, 16 components were screened and identified as the absorbed prototype constituents after oral administration of CH-BS, by comparing the retention time, precise molecular weight, fragmentation information of peaks, and the base peak chromatograms (BPC), which is shown in Figure 1. Among them, the peaks labeled 6, 7, 8, 11, 12, and 14 were identified as oxypaeoniflora, albiflorin, paeoniflorin, saikosaponin A, saikosaponin B_2, and saikosaponin D by comparing their retention times, MS, and MS/MS data with the authentic reference standard. For example, the compound corresponding to peak 8 (the retention time was 13.81 min) produced the quasi-molecular ion was *m/z* 479.1560 ($[M-H]^-$) and the ion at *m/z* 525.1617 was the adduction ($[M+HCOO]^-$) in negative ion mode. Besides, the main fragment ions were analyzed by the MS/MS data, and there were fragment ions *m/z* 449.1456 ($[M-CH_2O-H]^-$) formed by removing one molecule of formaldehyde and *m/z* 431.1373 ($[M-CH_2O-H_2O-H]^-$) formed by continuing to remove one molecule of water. Based on the HRMS data of peak 8 in serum samples of the CH-BS group, the molecular formula was determined to be $C_{23}H_{28}O_{11}$. Meanwhile, there were benzoyl characteristic ion *m/z* 121.0287 and pinane skeleton characteristic ion *m/z* 165.0550, which were consistent with the characteristic fragment ion of the paeoniflorin reference substance. Therefore, the compound corresponding to peak 8 was identified as paeoniflorin. Then, the peaks labeled 1, 2, 3, and 4 were identified as four monoterpene glycosides, which were from BS (desbenzoylpaeoniflorin, glucopyranosyl-paeonisuffrone, glucopyranosyl-lactinolide, mudanpioside F), the peaks labeled 10, 13, 15, and 16 were identified as four saponins from CH (saikosaponin C, acetyl-saikosapon A, acetyl-saikosaponin B_2, and acetyl-saikosaponin D), and the peaks labeled 5 and 10 were identified as gallic acid and ellagic acid, respectively, by comparing their retention times, accurate molecular weight (5 mDa error), and MS/MS data with the LC-MS data of standard compounds from the databases such as ChemFinder Ultra 14.0, ChemSpider (www.chemspider.com), and literature data.[16-18] As a result, a total of 16 absorbed prototype components of CH-BS herb pairs were identified, of which 7 constituents come from CH and 9 constituents come from BS.

Analysis of metabolites in serum samples

There were two metabolic reactions which were called phase I reactions and phase II reactions. The prototype components could be converted into aglycone, oxidized aglycone, or reduced aglycone by the phase I reactions. Besides, the phase II reactions were focused on conjugating with glucuronide and sulfate. In this study, the chromatographic peaks only appeared in the chromatogram of serum samples in the CH-BS group but not in the chromatograms of serum samples in the control group and extract samples of CH-BS were considered as metabolites. As a result, a total of 39 components were screened and identified as metabolites by analyzing their accurate molecular weight, MS/MS data, and literature data.[16-18] The available mass spectrometry information about the metabolites was listed.

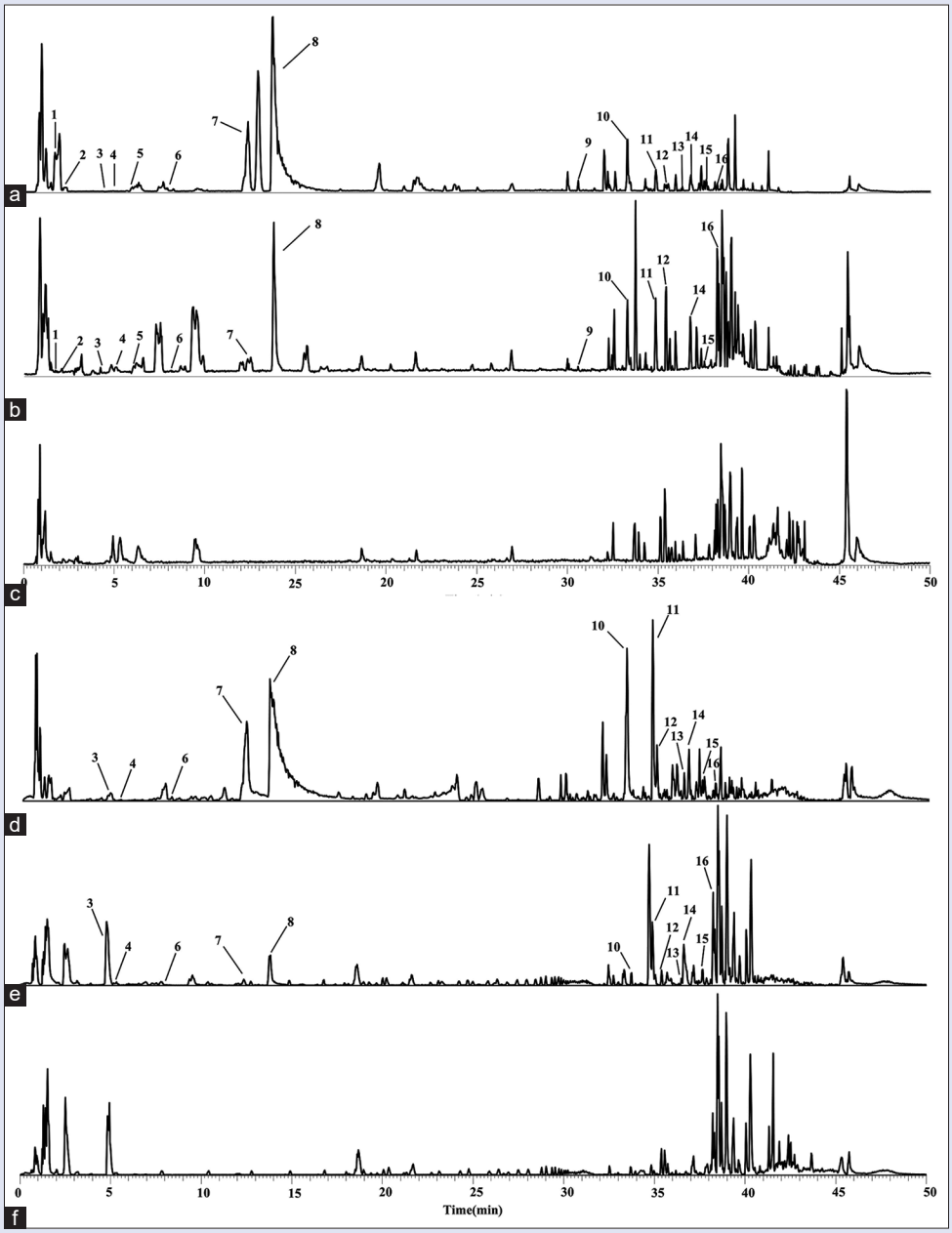

Figure 1: Base peak current chromatogram in positive (A ~ C) and negative (D ~ F) ion mode. (a and d) CH-BS herb pair extracts; (b and e) serum after administration of CH-BS herb pair; (c and f) blank serum. Peaks 1 ~ 16 are the absorbed prototype components, and corresponds to the serial number

Comparative analysis of the serum chemical profile of CH before and after compatibility based on background subtraction and metabolomics technology

Background subtraction algorithm of CH before and after compatibility

The background subtraction method is a technique to analyze LC-MS data by using the Strip tool to remove matrix-related signals from UHPLC-HRMS data. In this way, non-related ion signals could be effectively subtracted from LC-MS data of analyte samples, and those different ions between the background control samples and the analyte samples would be found and extracted for analysis.[19] In this study, using the background subtraction method, the LC/MS data of serum samples in the control group [Figure 2a] was subtracted from serum samples in the CH group [Figure 2b], to obtain the serum chemical profile of the CH before compatibility [Figure 2c]. Meanwhile, the LC-MS data of serum samples in the BS group [Figure 2d] was subtracted from serum samples in the CH-BS compatibility group [Figure 2e] to obtain the serum chemical profile of CH after compatibility without the interference of the ion signals from BS group samples [Figure 2f]. The results showed that the mass spectrometry data of the BS group were effectively subtracted from the CH-BS compatibility group by the background subtraction algorithm [Figure 2d-f].

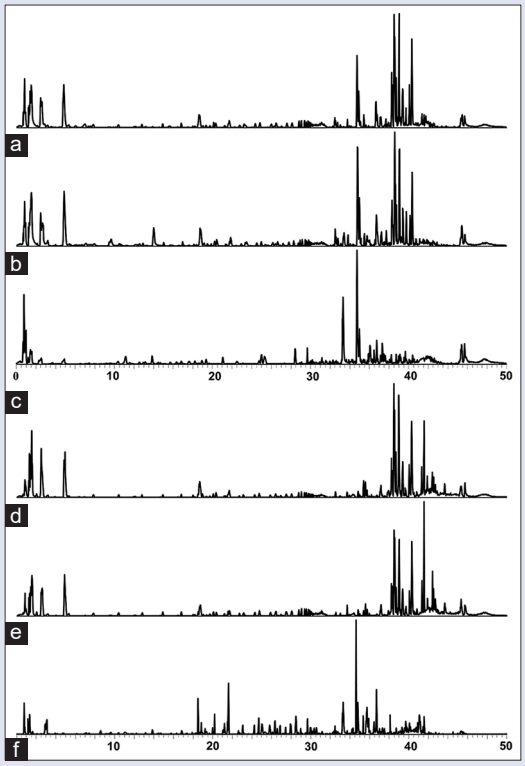

Figure 2: Comparative analysis of the serum chemical profile of CH before and after compatibility based on background subtraction algorithm. (a) Base peak ion chromatographic profiles of the control group rat's serum samples. (b) The CH group rat's serum samples. (c) The serum chemical profile of the CH before compatibility. (d) The BS group rat's serum samples. (e) The CH-BS group rat's serum samples. (f) The serum chemical profile of the CH after compatibility

Multivariate statistical analysis for screening the significantly differential chemical constituents *in vivo* before and after compatibility of CH

The LC-MS data of CH before and after compatibility based on the background subtraction algorithm were further imported into SIMCA-P 13.0 (Umetrics, Sweden) for multivariate statistical analyses, such as Principal components analysis (PCA), Partial least-squares discriminant analysis (PLS-DA), and Partial least-squares discriminant analysis (OPLS-DA). First, using the unsupervised PCA model, the PCA score plots indicated that the serum chemical profile of CH before compatibility could be clearly separated from that after compatibility [Figure 3a]. Then, the PLS-DA model was established, and the predictive ability of this multivariate statistical analysis model was verified by using the permutation plot test [Figure 3b]. The values of R^2X, R^2Y, and Q^2 of the model were 0.525, 0.998, and 0.962, respectively, indicating that the model had a good predictive ability. To further discover the difference in serum chemical profile of CH before and after compatibility, the OPLS-DA model was used. As shown in Figure 3c, the OPLS-DA score plots showed an obvious separation for the serum chemical profile of CH before and after compatibility [Figure 3c]. The differential components between before and after compatibility were screened by S-plots and VIP values in the OPLS-DA model [Figure 3d]. A total of 14 different features (10 components were identified and 4 components were unknown) were found according to the VIP values (VIP > 1.0) and *t*-tests ($P < 0.05$) [Table 1]. Besides, in the loading plot of PCA, these 14 different features have also been proved to have a large contribution and a higher confidence level to the difference between the two groups [Figure 3e]. In conclusion, compared with the administration of CH alone, three prototype components (saikosaponin A, saikosaponin D, and saikosaponin B_2) were significantly decreased and seven metabolites of prototype components (prosaikogenin F, prosaikogenin D, prosaikogenin G, hydroxy-saikogenin F,

Table 1: Differential components were screened and detected before and after the compatibility of CH and BS by the multivariate statistical analysis. "↓" or "↑" means the component significantly decreased or increased in the CH-BS herb pair group compared with CH or BS group. **P*<0.05, ***P*<0.01 vs CH or BS group

No.	Metabolites	t_R/min	*m/z*	Formula	VIP	Trend	Ion mode	Source or parent
P1	Desbenzoylpaeoniflorin	1.25	421.1341	$C_{16}H_{24}O_{10}$	1.69	↑**	$[M+HCOO]^-$	BS
P2	Glucopyranosyl-paeonisuffrone	1.94	405.1408	$C_{16}H_{24}O_9$	1.04	↑*	$[M+HCOO]^-$	BS
P3	Glucopyranosyl-lactinolide	4.65	407.1563	$C_{17}H_{21}O_{11}$	1.72	↑**	$[M-H]^-$	BS
P5	Methyl gallate	5.41	183.0296	$C_8H_8O_5$	1.73	↑**	$[M-H]^-$	BS
P6	Oxypaeoniflora	8.11	495.1512	$C_{23}H_{28}O_{12}$	1.10	↑*	$[M-H]^-$	BS
P7	Albiflorin	12.26	525.1619	$C_{23}H_{28}O_{11}$	1.01	↑*	$[M+HCOO]^-$	BS
P8	Paeoniflorin	13.81	525.1616	$C_{23}H_{28}O_{11}$	3.53	↑**	$[M+HCOO]^-$	BS
P9	Dimethyl-ellagic acid	30.51	329.0306	$C_{16}H_{10}O_8$	1.53	↑*	$[M-H]^-$	BS
P11	Saikosaponin A	34.83	779.4597	$C_{42}H_{68}O_{13}$	1.69	↓**	$[M-H]^-$	CH
P12	Saikosaponin B2	35.42	779.4592	$C_{42}H_{68}O_{13}$	1.74	↓**	$[M-H]^-$	CH
P14	Saikosaponin D	36.73	779.4591	$C_{42}H_{68}O_{13}$	1.72	↓**	$[M-H]^-$	CH
M1	Methylcatechin-5-O-glucuronide	9.26	479.1205	$C_{22}H_{24}O_{12}$	1.96	↑**	$[M-H]^-$	Catechin
M4	3-Hydroxy-methoxyl phenylpropionic acid sulfate	7.59	275.0234	$C_{10}H_{12}O_7S$	1.03	↑*	$[M-H]^-$	Catechin
M10	Methylgallic acid glucuronide	9.06	359.0551	$C_{14}H_{16}O_{11}$	1.13	↑*	$[M-H]^-$	Gallic acid
M11	Methylgallic acid sulfate	4.07	262.9866	$C_8H_8O_8S$	1.04	↑*	$[M-H]^-$	Gallic acid
M14	Pyrogallol sulfate	10.47	204.9812	$C_6H_6O_6S$	1.82	↑**	$[M-H]^-$	Gallic acid
M15	Methylpyrogallol sulfate	2.97	218.9933	$C_7H_8O_6S$	1.10	↑*	$[M-H]^-$	Gallic acid
M17	Paeonimetabolin I	18.20	197.0815	$C_{10}H_{14}O_4$	1.12	↓*	$[M-H]^-$	Paeoniflorin
M22	Prosaikogenin F	34.29	662.4034	$C_{36}H_{57}O_8$	1.73	↑**	$[M+HCOO]^-$	Saikosaponin A
M23	Prosaikogenin D	35.84	662.4035	$C_{36}H_{57}O_8$	1.10	↑*	$[M+HCOO]^-$	Saikosaponin B_2
M24	Prosaikogenin G	38.16	662.4033	$C_{36}H_{57}O_8$	1.24	↑*	$[M+HCOO]^-$	Saikosaponin D
M29	Hydroxy-saikogenin F	32.70	471.3471	$C_{30}H_{48}O_5$	3.53	↑**	$[M-H_2O+H]^+$	Saikosaponin A
M30	Dihydroxyl-dehydrogenation-saikogenin F	29.93	485.3261	$C_{30}H_{46}O_6$	1.96	↑**	$[M-H_2O+H]^+$	Saikosaponin A
M38	Trihydroxyl-dehydrogenation-saikogenin E	30.79	485.3270	$C_{30}H_{46}O_6$	1.03	↑**	$[M-H_2O+H]^+$	Saikosaponin C
M39	Trihydroxyl-saikogenin E	29.79	487.3490	$C_{30}H_{48}O_6$	2.03	↑**	$[M-H_2O+H]^+$	Saikosaponin C

P: prototype components absorbed into the serum; M: metabolites of prototype components; CH: *Radix Bupleuri*; BS: Radix Paeoniae Alba

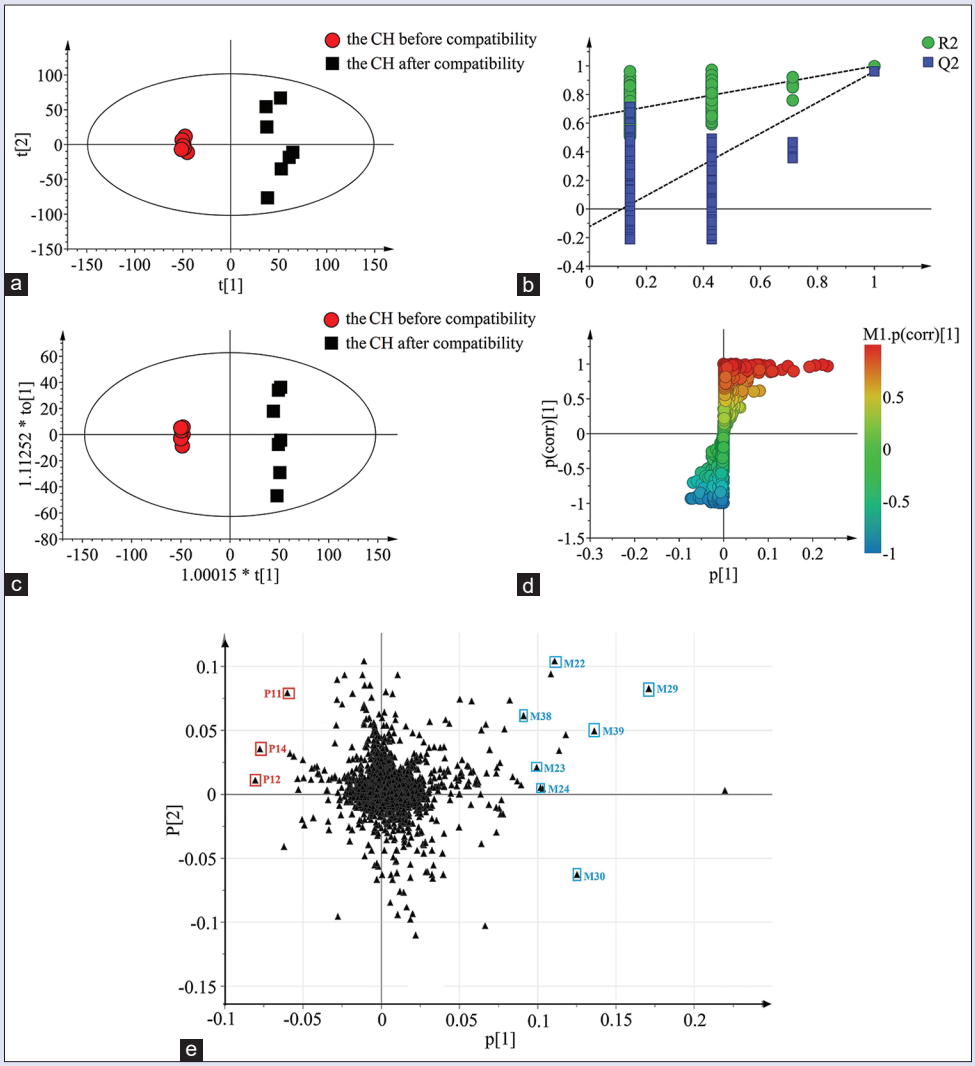

Figure 3: Multivariate statistical analysis of UPLC–MS data in serum dosed with CH alone-use group or compatibility group. (a) PCA score plots from the CH before and after compatibility. (b) PLS-DA model validation diagram. (c) OPLS-DA score plots from the CH before and after compatibility. (d) S-plot of OPLS-DA. (e) Loading plot of PCA in both positive and negative modes. The red square indicates the difference between prototype components; The blue square indicates the difference in the metabolites of prototype components. The differential components represented by the numbers in the figure match the numbers in Table 1

trihydroxyl-saikogenin E, dihydroxyl-dehydrogenation-saikogenin F, and trihydroxyl-dehydrogenation-saikogenin E) were significantly increased in the CH-BS compatibility group [Figure 4] Moreover, four unknown components were also significantly increased in the CH-BS compatibility group compared with CH alone. These results indicated that compatibility can enhance the metabolism of the CH prototype components *in vivo*.

Comparative analysis of the serum chemical profile of BS before and after compatibility based on background subtraction and metabolomics technology

Background subtraction algorithm of BS before and after compatibility

Using the background subtraction method, the LC/MS data of the control group rat's serum samples were subtracted from the BS group rat's serum samples, to obtain the serum chemical profile of BS before compatibility. The results showed that the non-related ion signals of endogenous matrix components from the control group rat's serum could be effectively subtracted. Besides, for the serum chemical profile of BS after the compatibility, the CH group rat's serum samples were used to provide background, and the LC/MS data of the CH group rat's serum samples was subtracted from the CH-BS compatibility group rat's serum samples based on background subtraction algorithm, to obtain the serum chemical profile of BS after compatibility. The results showed that the LC/MS data of the CH group were effectively subtracted from the CH-BS compatibility group, and the serum chemical profile of BS after compatibility was successfully obtained without the interference of the non-related ion signals from the CH group rat's samples.

Multivariate statistical analysis for screening the significantly differential components *in vivo* before and after compatibility of BS

After the acquisition of the background-subtracted data from UHPLC-HRMS, the PCA model was applied to find out the serum chemical profile distinction in BS alone-use group and compatibility

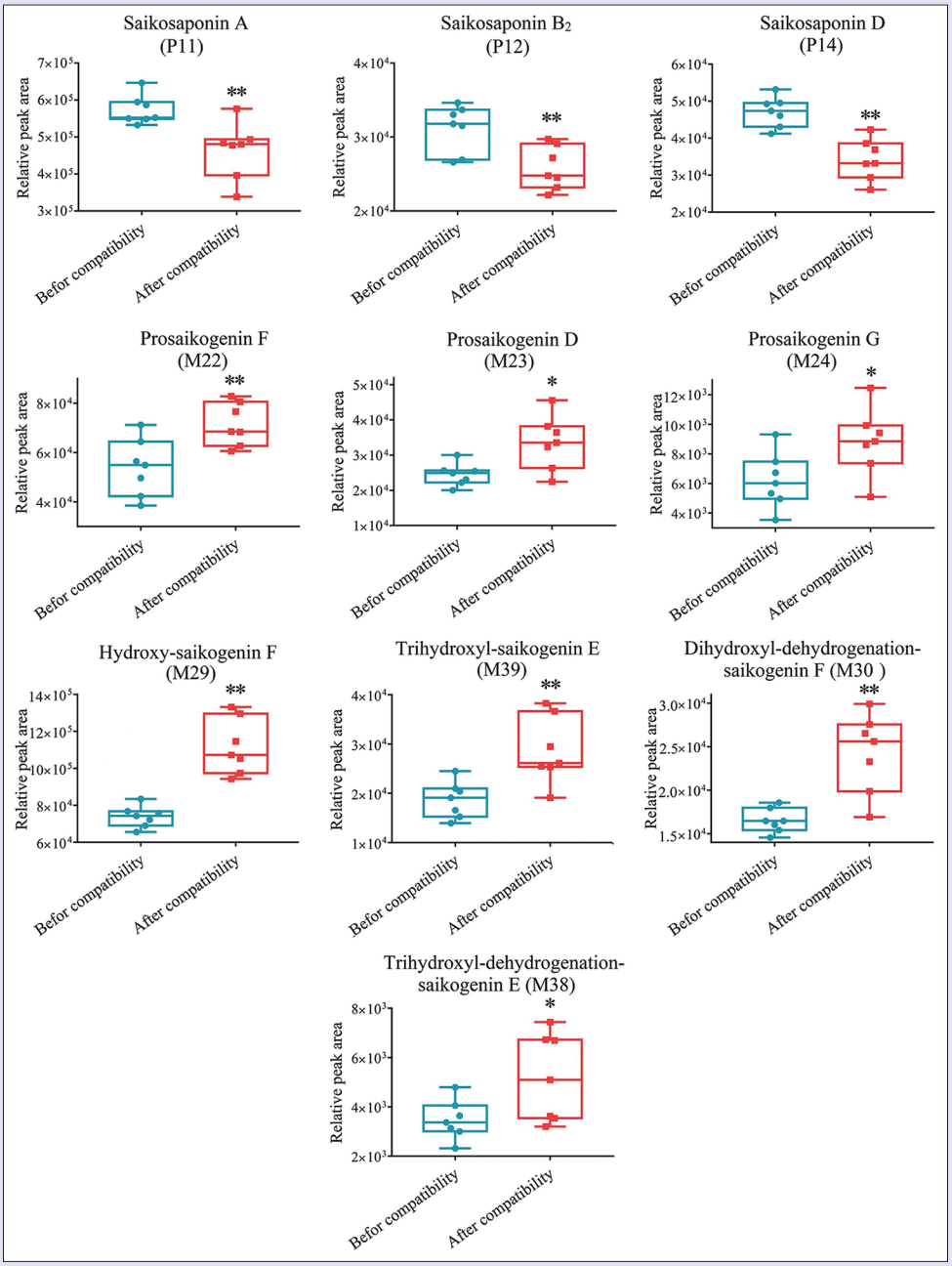

Figure 4: Comparison of relative peak areas of the differential components in CH alone-use group and compatibility group. All of the data were expressed as mean ± SD, (n = 7). **P* < 0.05, ***P* < 0.01, compared with CH alone-use group

group. The PCA score plots results showed that the BS alone-use group could be separated from the BS after the compatibility group [Figure 5a]. Moreover, the permutation test parameters (R^2X, R^2Y, and Q^2) of the PLS-DA model were 0.550, 0.997, and 0.996, respectively, suggesting that the multivariate statistical analyses model had an excellent predictive ability [Figure 5b]. The OPLS-DA model was used to improve the ability of differential components discovery in BS alone-use group and compatibility group rat's serum [Figure 5c]. The differential components were screened by the S-plot [Figure 5d] and VIP value, and VIP >1 with $P < 0.05$ was considered. At last, a total of 20 different features were screened by the VIP values (VIP > 1.0) and *t*-tests ($P < 0.05$), including eight prototype components, seven metabolites of prototype components, and five unknown components [Table 1]. Besides, these 20 different features have also been proved to have a large contribution and a higher confidence level to the difference between the BS alone-use group and compatibility group in the loading plot of PCA [Figure 5e]. Specifically, after administration of CH-BS, eight prototype components (desbenzoylpaeoniflorin, glucopyranosyl-paeonisuffrone, glucopyranosyl-lactinolide, methyl gallate, oxypaeoniflora, albiflorin, paeoniflorin, dimethyl-ellagic acid) and six metabolites of prototype components (methylcatechin-5-O-glucuronide, 3-Hydroxy-methoxyl phenylpropionic acid sulfate, methylgallic acid glucuronide, methylgallic acid sulfate, pyrogallol sulfate, methylpyrogallol sulfate) were significantly increased, and the one metabolite (paeonimetabolin I) was significantly decreased [Figure 6]. These results indicated that

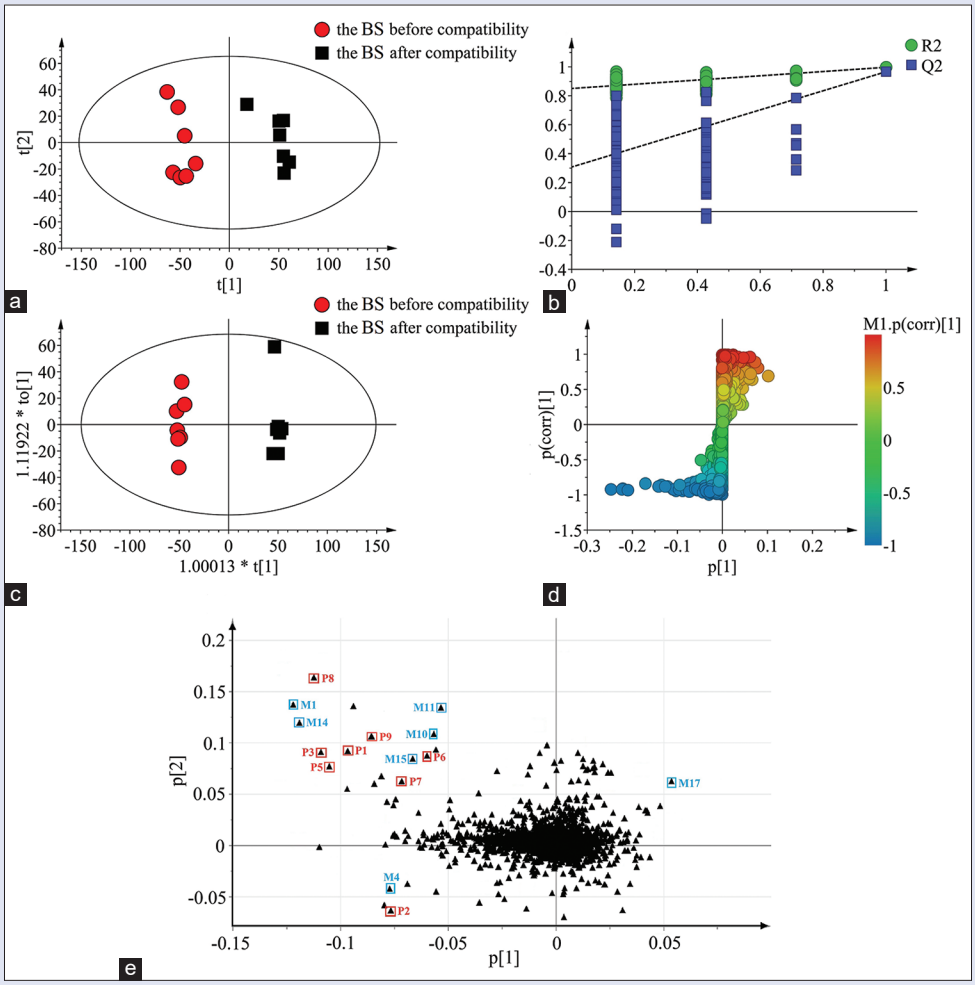

Figure 5: Multivariate statistical analysis of UPLC–MS data in serum dosed with BS alone-use group or compatibility group. (a) PCA score plots from the BS before and after compatibility. (b) PLS-DA model validation diagram. (c) OPLS-DA score plots from the BS before and after compatibility. (d) S-plot of OPLS-DA. (e) Loading plot of PCA in both positive and negative modes. The red square indicates the difference in prototype components; The blue square indicates the difference in the metabolites. The differential components represented by the numbers in the figure match the numbers in Table 1

compatibility can enhance the absorption of the prototype components in BS.

DISCUSSION

The herb-herb compatibility was the embodiment of the wisdom of TCM. To clarify the scientific connotation of herb-herb compatibility is of special significance for the advancement of the development of TCM. Multivariate statistical analysis and LC-MS have been widely applied to analyze and investigate potential pharmacodynamic markers from TCM,[20] but interference from biological matrices is still the main challenge for the detection of metabolic objects.[21] Moreover, how to remove the interference of the mass spectrum information of one herb for another herb is still a difficult problem in the study of herb-herb compatibility.[22] In this present study, we developed a reliable method to analyze the difference in serum chemical profile before and after the compatibility of CH and BS by the background subtraction algorithm. First, using the background subtraction method, the LC/MS data of the control group rat's serum samples were subtracted from the CH (or BS) group rat's serum samples, to obtain the serum chemical profile of the CH (or BS) before compatibility. Besides, the LC-MS data of the CH (or BS) group rat's serum samples were subtracted from the CH-BS compatibility group rat's serum samples, to obtain the serum chemical profile of CH (or BS) after compatibility. Our present results suggested that the non-related ion signals of endogenous matrix components from the control group rat's serum could be effectively subtracted, and these target signals come from CH (or BS) before compatibility could be effectively amplified. For the serum chemical profile of the CH (or BS) after compatibility, the LC/MS data of the BS (or CH) group and the endogenous matrix components were effectively subtracted from the CH-BS compatibility group, and the serum chemical profile of CH (or BS) after compatibility was successfully obtained based on the background subtraction algorithm.

Multivariate statistical analysis is a key technology in metabolomics research. It can not only identify small molecular endogenous metabolites in the body but also as a valuable tool for analyzing the complex components and metabolites of herbal medicine in biofluids. With the help of this tool, the serum chemical profile information of chemical components in complex herbal medicine could be effectively mined.[23] In our research, the multivariate statistical analysis results revealed that the compatibility of CH and BS can significantly reduce the concentration of saikosaponins, and increase the concentration of saikosaponins metabolites, which indicated that compatibility can enhance the metabolism of the CH prototype constituents *in vivo*. Meanwhile, the compatibility can also enhance the absorption of

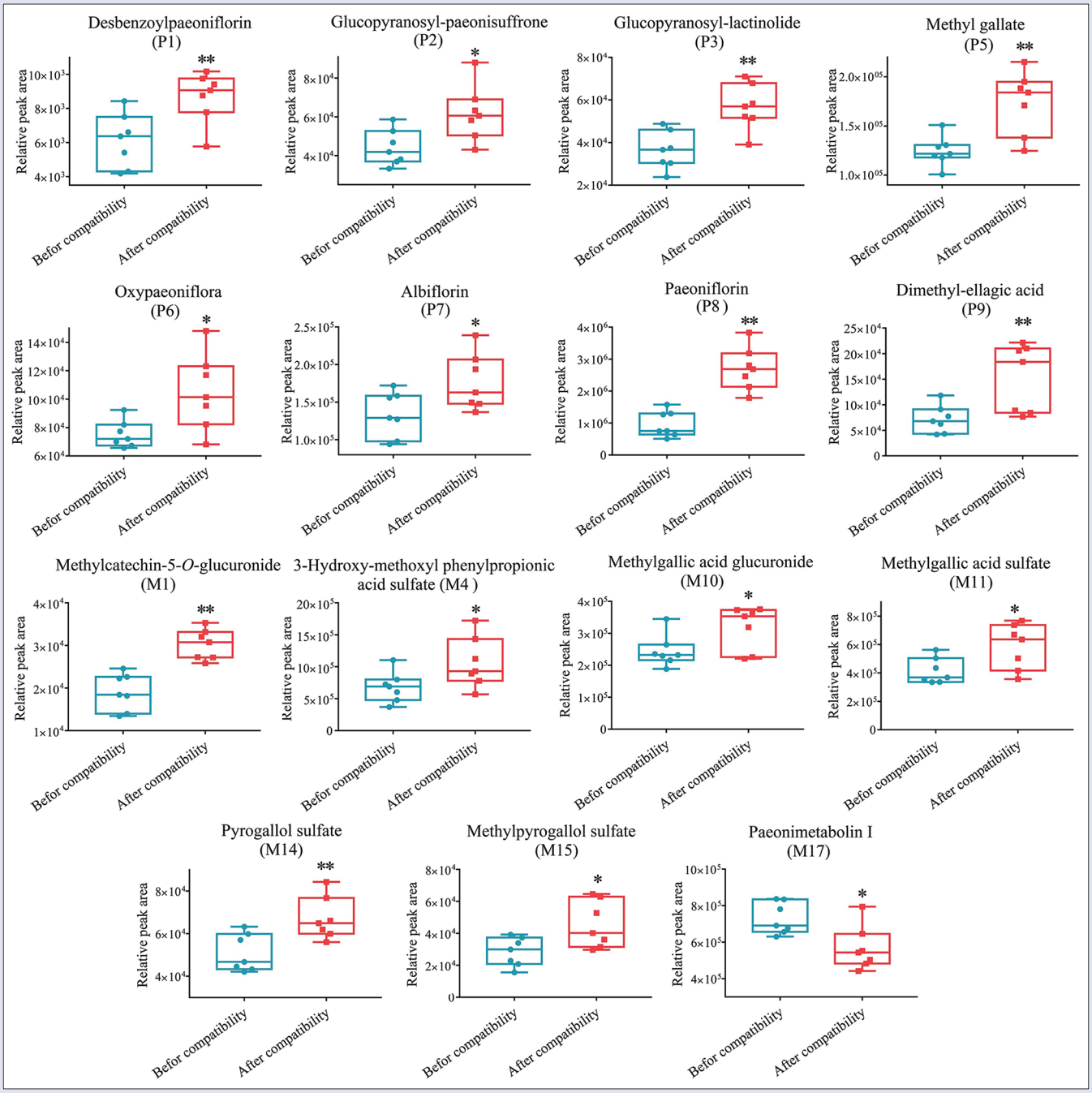

Figure 6: Comparison of relative peak areas of the differential components in BS alone-use group and compatibility group. All of the data were expressed as mean ± SD, (n = 7). *$P < 0.05$, **$P < 0.01$, compared with BS alone-use group

the prototype constituents in BS. Previous studies have shown that saikosaponin A and saikosaponin D could significantly improve the absorption of paeoniflorin in the ileum and colon,[23] which could be the possible reason for the compatibility of CH and BS that can enhance the absorption of the prototype constituents in BS.

In this present study, we developed a reliable method to discover, screen, and analyze multiple absorbed bioactive ingredients and metabolites in complex Chinese medicine ingredients. After the oral administration of CH-BS, a rapid and sensitive method based on UHPLC-QE-Orbitrap-MS was established to analyze and identify the chemical composition and metabolites of rats *in vivo*. Most importantly, this work applied the background subtraction method to herb-herb compatibility research for the first time. Interference was effectively removed from biological matrices and non-research target drug pairs. Through the combination of the background subtraction method and metabolomics, we found that the application of the CH-BS herb pair could remarkably promote the *in vivo* conversion of saponin components in CH, and also obviously enhance the blood absorption of the prototype components in BS. The identification and structural elucidation of this chemical compound provided necessary data for further pharmacological studies of CH-BS, and this research method

can be regarded as the technological reference to other researches on the mechanism of herb pair compatibility.

CONCLUSION

An effective strategy has been developed for screening differences of constituents *in vivo* before and after compatibility by combining UHPLC-QE-Orbitrap-MS based on background subtraction and metabolomics. The established methodology also could be widely applied for the global characterization of constituents before and after compatibility in TCM.

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Conflicts of interest

There are no conflicts of interest.

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