



Application of pseudotargeted method combined with multivariate statistical analysis for the quality assessment of traditional Chinese medicine preparation, Sanhuang Tablet as a case

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Abstract

The quality control of traditional Chinese herbal medicine (TCHM) is considered to be one of the constraints for its modernization and globalization, especially for traditional Chinese medicine preparation (TCMP). The active compounds at high concentration in TCMP are usually selected as markers for qualification and quantification analyses, but we thought these compounds are insufficient to represent the quality of TCMP. For the in-depth investigation, a pseudotargeted analysis method using ultra-high-performance liquid chromatography-quadrupole-orbitrap mass spectrometry operated in the parallel reaction monitoring mode with time-staggered ion list (tsPRM) was proposed to recognize the chemical composition of TCMP. The full scan-based untargeted analysis was carried out to obtain the target ions. After data preprocessing, the target ions were extracted and to be used as inclusion lists for subsequent parallel reaction monitoring (PRM) analysis. The established pseudotargeted method exhibited good repeatability and wide linear range. Moreover, the number of scan points for the ions in the high coelution zone was greatly increased with the help of tsPRM mode, which makes the compound quantification accuracy. Then, the established method was successfully applied to discover analytical markers for Sanhuang Tablet, which demonstrates its applicability and great potential in quality assessment of TCMP.

Keywords Pseudotargeted analysis · Parallel reaction monitoring · Traditional Chinese medicine preparation · Multivariate statistical analysis

Introduction

Traditional Chinese herbal medicine (TCHM) has been attracting growing research interests all over the world, due to its holistic body adjustment theory, long historical clinical practice, and minimal side effect. The quality control of TCHM is one of the bottlenecks of its modernization and globalization, especially for traditional Chinese medicine preparation (TCMP) [1, 2]. Conventionally, the components that have certain bioactivities with high concentrations are

selected as analytical markers to perform qualitative or quantitative analysis. However, the highly complex chemical composition and multi-targeted bioactivity of TCMP makes it unable to be completely characterized by a limited number of components. Moreover, since the chemical transformation or degradation of components occurs in the manufacturing procedure, the markers of a particular herb may not be suitable for quality control of TCMP [3–6]. Hence, the comprehensive approaches that enable comprehensive and reliable data acquisition for qualification and quantification should be developed to recognize analytical markers for the quality assessment of TCMP.

The untargeted analysis method could comprehensively detect the components present in the test samples without any prior knowledge of these features using high-resolution mass spectrometers (HRMS) [7, 8]. But the full scan detection usually suffers from the narrow linear range caused by matrix effects or detector saturation, which poses a challenge to the quantitative analysis of components in a wide concentration range. Targeted analysis method measures a limited number

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of chemically characterized and annotated components by means of multiple reaction monitoring (MRM) performed on a triple quadrupole (QQQ) MS instrument, which possesses wide linear dynamic range, high sensitivity, and high repeatability [9, 10]. But the requirement of standard references during analysis greatly decreases its detection coverage, making this method is not able to obtain information comprehensively. The emergence of the pseudotargeted method allows combining the merits of untargeted and targeted methods via using two MS instruments, in which HRMS was applied to obtain MS/MS information of component under data-dependent acquisition (DDA) initially; then, the precursor-fragment ion pairs were extracted and to be monitored by a QQQ MS under MRM mode [11]. Much effort has been devoted to improving this method, including to increase the MS/MS coverage and simplify the ion pair selection, which greatly expand the application of this approach [12–15], whereas two MS instruments are still needed to complete the analysis. With the introduction of MRM-like functionality in HRMS, an improved pseudotargeted method was established using a Q-TOF MS under multiple ion monitoring (MIM) acquisition mode [16], but the lack of MS/MS information make it difficult for component identification, and the quantification accuracy should be influenced. The parallel reaction monitoring (PRM) acquisition possesses the advantages of acquiring MS and MS/MS information of analyte in one analytical run parallelly [17, 18], which allows to components' confirmation and quantification by examining the tandem MS spectra, suggesting that PRM has potential as a viable alternative in pseudotargeted analysis.

Herein, a pseudotargeted method was proposed to analyze the components of TCMP by using ultra-high-performance liquid chromatography-quadrupole-orbitrap mass spectrometry (UHPLC-Q-Orbitrap/MS). The untargeted analysis was performed to characterize the components in a pooled TCMP extract. Target ions extracted from raw data were then monitored by the same UHPLC-Q-Orbitrap/MS operated on parallel reaction monitoring (PRM) mode to achieve the pseudotargeted analysis. PRM with time-staggered ion lists (tsPRM) was introduced to increase the number of the scan point of ion peaks, and further to ensure data quality and the accuracy of quantification. Sanhuang Tablet (SHT) is a classical TCMP that consists of Rhei Radix et Rhizoma, Scutellariae Radix extract, and berberine hydrochloride and was employed as a proof-of-concept to verify the applicability of the established method. After PRM acquisition, the extract chromatogram of quantitative ion of each feature was integrated, and the peak area was used to conduct the statistical analysis, including partial least squares-discriminant analysis (PLS-DA) and *t* test to find analytical markers for the quality assessment of SHT. Due to its potential for comprehensively acquiring qualitative and quantitative information of given samples, it is expected that the established pseudotargeted

method can serve as a practical tool for component profiling of TCMP.

Material and methods

Chemicals and materials

HPLC-grade acetonitrile, methanol, and formic acid were purchased from TEDIA (Fairfield, OH, USA). Ultrapure water was filtered through a Milli-Q system (Millipore, Billerica, MA, USA). Ten SHT samples produced by two different manufactories (M1 and M2), respectively, were purchased from the local market. Standard references of baicalin, berberine, baicalein, wogonin, emodin, and chrysophanol were obtained from Nanjing Zelang Co., Ltd. (purity \geq 98%, Nanjing, China).

Sample preparation

The coatings of SHT samples were removed completely, and the remainder were crashed into the fine powder. The pulverized sample (100 mg) of each sample was weighed precisely; then, 50 mL methanol was added for 30 min of ultrasonic extraction. The supernatant was filtered by a 0.22- μ m filter for LC-MS analysis. A pooled "quality control" (QC) sample was prepared by mixing equal aliquots (50 μ L) from all the filtered supernatants of SHT samples for the method validation. The QC sample was analyzed three times initially to ensure system equilibration and once every four samples during analytical run; the gradient diluted QC sample was prepared to test the linearity of the method.

The standard references (baicalin, berberine, emodin, and chrysophanol) were accurately weighed and dissolved in methanol to prepare a stock solution. Stock solutions of the mixed standards were diluted to a variety of different concentrations to construct the calibration curves via plotting the peak areas of quantitative ions versus the concentrations of the corresponding constituents. All the prepared solutions were stored at 4 °C.

UHPLC-Q-Orbitrap/MS analysis

The chromatographic separation was performed on an UltiMate 3000 system (Dionex, Sunnyvale, CA, USA) coupled with ACQUITY UHPLC BEH C18 column (2.1 \times 100 mm, 1.7 μ m; Waters, Milford, MA, USA) maintained at 60 °C. Mobile phase A was 0.1% formic acid in the water, and mobile phase B was 100% acetonitrile. The proportion of acetonitrile gradient was used as follows: 5% (0–1 min), 5–30% (1–2 min), 30–70% (2–13 min), 70–100% (13–13.5 min), and then back to 5% at 13.6 min for 2.9 min of

equilibration. The injection volume was set to 2.0 μL , and the flow rate was set to 0.4 mL/min.

Mass spectrometric detection was implemented on a Q-Exactive Orbitrap/MS (Thermo, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source operated in positive ion mode. The key parameters of ionization source were set as follows: sheath gas flow of 45 Arb, aux gas flow of 13 Arb, and sweep gas flow of 1 Arb; capillary voltage was set to +3.5 kV at the capillary temperature of 342 °C and aux gas heater temperature of 358 °C; the S-Lens RF level was 70%. Full scan MS data were acquired from m/z 100 to 1000 in centroid mode, using the resolution of 70,000 with automatic gain control (AGC) target of 1×10^6 and maximum injection time (IT) of 100 ms. The MS/MS data were acquired in PRM mode using the following settings: resolution 17,500 with AGC target of 1×10^5 , maximum IT of 50 ms, and the normalized collision energy (NCE) of 25–45%. The mass range of MS/MS spectra varies depending on the precursor ions.

Data processing and statistical analysis

After full scan-based untargeted data acquisition, the raw data was imported into Sieve software (version 2.1, Thermo, San Jose, CA, USA) to perform data preprocessing, including background subtraction, component detection, peak alignment, and ion fusion. The base peak minimum intensity and background were set at 10^5 and 3, respectively. The maximum retention time shift was set at 0.2 min and the m/z width was 10 ppm to align the features extracted from each chromatogram. The ion fusion was performed to reduce redundant interference ions, e.g., adduct ions ($[\text{M}+\text{H}]^+$, $[\text{M}+\text{NH}_4]^+$, $[\text{M}+\text{Na}]^+$, and $[\text{M}+\text{K}]^+$) and fragmentation ions (neutral loss of H_2O and CO_2). Then, the target ion list was obtained from the untargeted LC-MS analysis using the above procedures and was transferred into the inclusion ion list of PRM acquisition mode to perform pseudotargeted analysis using UHPLC-Q-Orbitrap/MS. The monitored window for each target ion was defined as retention time (RT) \pm 0.5 min, and the ramped collision energy was applied to obtain fragment ions. The tsPRM detection was used to ensure the data quality, in which the inclusion ion list was divided into three separate sublists based on the staggered time points following the reported procedure [16]. In short, the extracted target ions were sorted by retention time in ascending order; then, these ions were labeled with repetitive serial numbers (e.g., 1, 2, 3, 1, 2, 3...) based on the number of the sublist to be established; finally, the ions with the same serial number were collected to construct a new ion list. After PRM acquisition, the product ion with the highest intensity in tandem mass spectrum of each target ion was selected as quantitative ion to conduct the peak extraction and peak area integration for each feature. And the resulted peak area of each component in QC sample was used

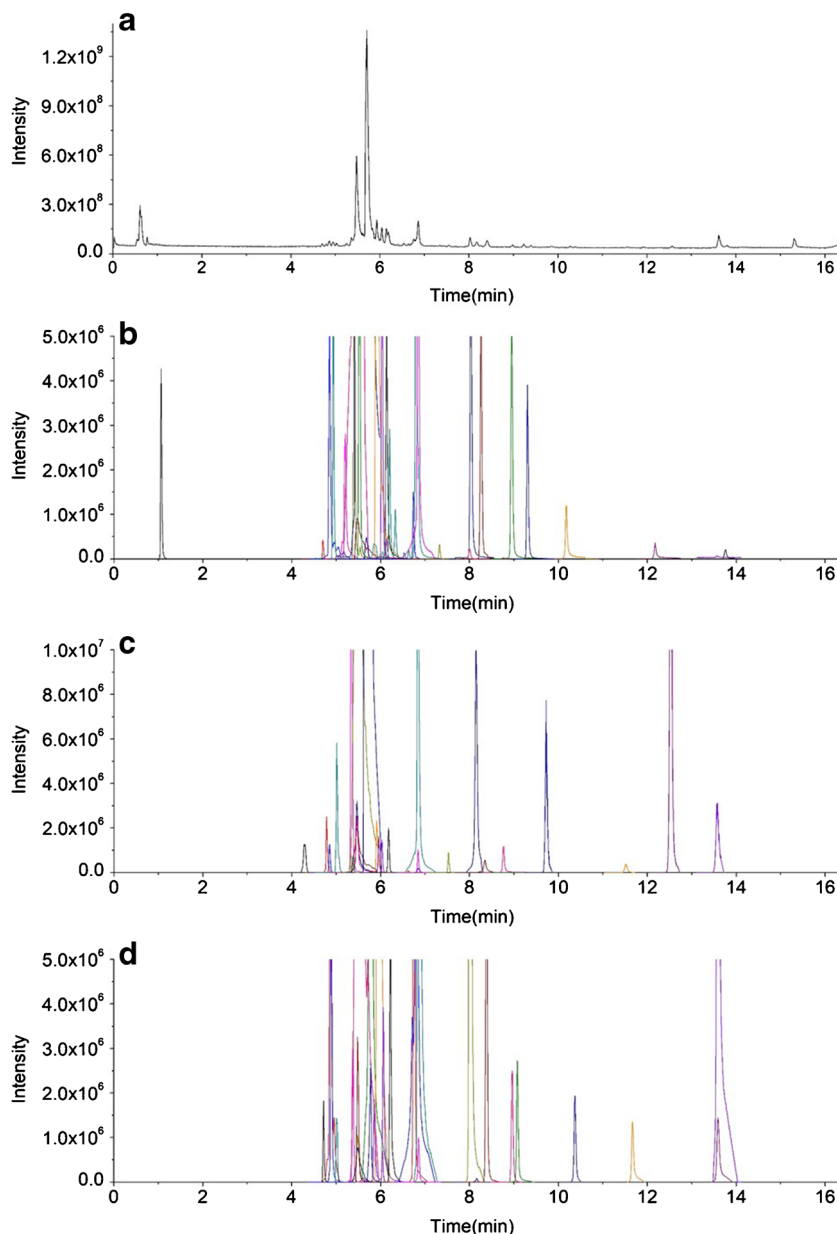
to evaluate the repeatability and linearity of the established method. The discrimination of SHT samples was implemented through the multivariate statistical analysis of detected peak area of each sample using SIMCA software 13.0 (Umetrics, Umea, Sweden). Student's t test was used to assess the significant difference of analytical markers between groups by using SPSS19.0 (Chicago, IL, USA). A value of $p < 0.05$ was considered to be a significant difference.

Results

Construction of the pseudotargeted analysis method

During PRM analysis, a duty cycle is usually initiated by an MS survey scan, and then, a series of targeted MS/MS experiments were performed. The MS survey collects high-resolution mass spectra comprehensively, which allows for profiling of all precursors across a certain m/z range. After isolating a preset precursor ion in the quadrupole, the MS/MS acquisition in PRM mode was conducted via detecting all product ions generated from collision-induced dissociation. PRM on Orbitrap/MS platforms enables targeted monitoring of the precursor ion and recording all the fragment ions in parallel, which allows to components' confirmation and quantification by examining the tandem MS spectra. Therefore, the PRM analysis was introduced to establish the present pseudotargeted analysis method using UHPLC-Q-Orbitrap/MS, and the typical workflow was shown in Fig. S1 in the [Electronic Supplementary Material](#) (ESM). Full scan-based untargeted analysis of QC samples was conducted to acquire MS spectra of the components to ensure the detection of a wide range of components in TCMP sample. As shown in Fig. 1a, the total ion chromatogram (TIC) of SHT QC sample obtained in full scan shows well separation within 16.5 min. After being processed by Sieve software, 85 ions were extracted as target ions and imported into the inclusion list to perform subsequent PRM analysis. Then, quantitative ions were selected by inspecting the MS/MS spectra obtained from PRM analysis to perform peak extraction and peak area integration. Specifically, the fragment ion with the highest intensity in MS/MS spectrum was selected as quantitative ion. Here the ion of wogonin at RT 8.03 min m/z 285.08 was taken as an example to demonstrate the procedure; the protonated wogonin ion at m/z 285.08 was the base peak in the full scan mass spectrum and was chosen as the precursor ion (ESM Fig. S2A). After PRM acquisition, the MS/MS spectrum (ESM Fig. S2B) showed that the ion at m/z 270.05 corresponding to the $[\text{M}+\text{H}-\text{CH}_3]^+$ ion has the highest intensity and was selected as the quantitative ion. Then, the extract chromatogram of quantitative ion was integrated, and the peak area was used to conduct the subsequent quantification analysis.

Fig. 1 Representative chromatograms of SHT QC sample. Total ion chromatogram obtained by full scan (**a**), and the constructed tsPRM chromatogram of pseudotargeted analysis obtained from three separate target ion lists (**b–d**)



As shown in Fig. 2, the heterogeneous distribution of the selected target ion was observed, which is commonly occurred in LC-MS analysis, due to there is a group of compounds with similar structures or polarities that usually have similar RT and m/z value. The data acquisition rate of Q-Orbitrap/MS was 3 spectra per second at full scan mode with 70,000 resolution and 12 spectra per second at PRM mode with 17,500 resolution. Therefore, several compounds may suffer from fewer scan points during PRM acquisition in the high coelution zone (e.g., RT 4.2–8.4 min in Fig. 2), which will affect the accuracy of its quantification. To address this issue, the tsPRM by dividing the original target ion list into three time-staggered ion lists were introduced to perform the pseudotargeted analysis. By means of tsPRM, the number of

scan point for the ion peak was greatly increased. Two components at RT 4.71 m/z 271.06 and RT 4.71 m/z 455.09 in the high coelution area were selected to illustrate. As shown in Figs. 3, 7, and 5, scan points were obtained for these two ions in the PRM analysis, respectively. After introducing the tsPRM ion list, the scan points were increased to 19 and 21, respectively, which ensures the accuracy of their quantification.

Repeatability and linearity of the pseudotargeted analysis method

Representative extracted chromatogram tsPRM analysis is shown in Fig. 1b and c. The constructed tsPRM

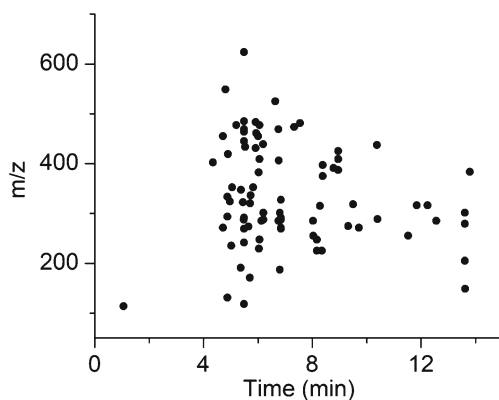


Fig. 2 The distribution of the target ions

chromatogram exhibited the similar peak profile with the TIC detected in full scan analysis, but the signal to noise ratio of peaks, especially for the ions with low intensity in TIC, was greatly improved.

The repeatability and linearity of the established pseudotargeted method were evaluated using the peak area of 85 peaks obtained from the untargeted analysis. To assess the repeatability of both methods, the pooled QC sample was repeatedly analyzed 7 times under full scan and tsPRM modes, respectively. Then, the relative standard deviations (RSDs) and the distribution of the RSDs of the peak areas of the 85 ions detected in each mode were calculated to evaluate the method repeatability. As shown in Fig. 4a, 63.6% of the

components detected by tsPRM exhibited an RSD of less than 5%, which was over seven times higher than that detected in full scan analysis. Furthermore, almost 95.3% of the tsPRM-detected components had an RSD of less than 20%, suggesting the great repeatability of the tsPRM-based pseudotargeted method for components analysis.

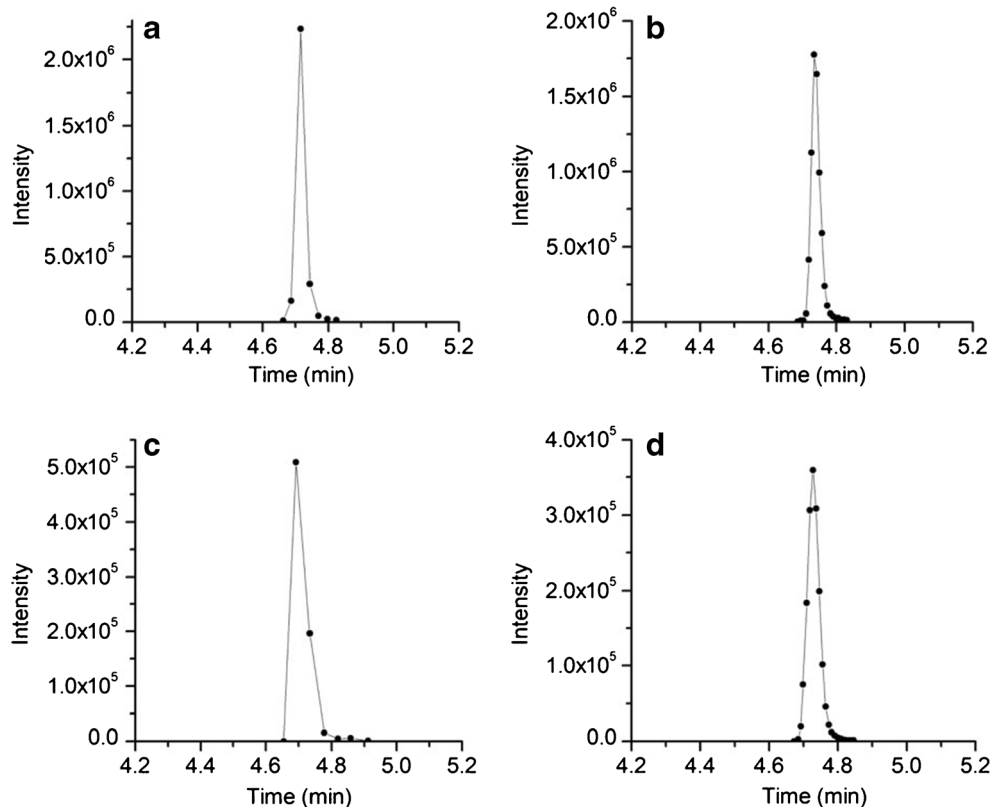
For the linearity evaluation, due to the limited number of standards and incomplete identification of compounds, the gradient diluted QC samples were used as dummy standards to be analyzed by the UHPLC-Q-Orbitrap/MS under full scan and tsPRM modes, respectively. The correlation between the peak areas and the concentrations of the components in the SHT sample was evaluated by the linear correlation coefficient (r). As shown in Fig. 4b, 51.8% and 10.1% of the compounds detected under the tsPRM and full scan modes have a correlation coefficient larger than 0.999, respectively. And 69.4% of tsPRM-detected and 23.9% of full scan-detected compounds had an r value larger than 0.99.

Compared with full scan analysis, the results of the method validation indicated that the developed UHPLC-Q-Orbitrap/MS tsPRM MS-based pseudotargeted method has better repeatability, higher sensitivity, and wider linear range.

Quantification analysis of SHT

Baicalin, berberine, emodin, and chrysophanol are the main active components in SHT and have been selected as

Fig. 3 Extracted ion chromatograms of ions at RT 4.71 min, m/z 271.06 and RT 4.71 min, m/z 455.09 acquired from PRM (a, c) and tsPRM (b, d) modes



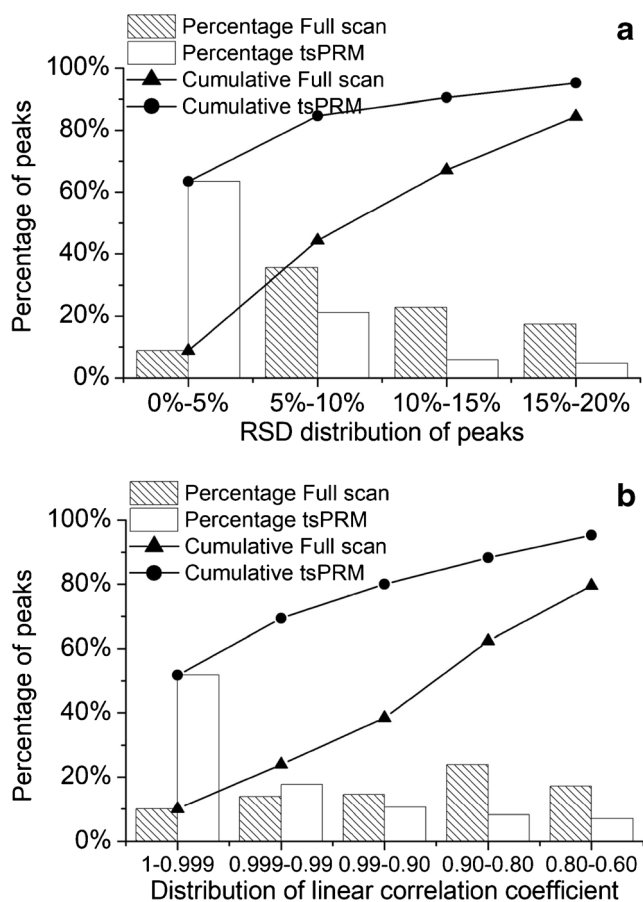


Fig. 4 Repeatability ($n=7$) (a) and linear response (b) of components detected in the full scan and tsPRM modes, respectively

analytical markers for its qualification and quantification as recorded in Pharmacopoeia of the People's Republic of China (version 2015). Thus, these four compounds were quantified using UHPLC-Q-Orbitrap/MS in this study. The calibration curves are shown in Table 1, in which all compounds show good linearity with an r value larger than 0.99, and the limit of detections (LODs, $S/N=3$) of all compounds was also calculated. Good precision and accuracy of this method were obtained by repeatedly analyzing these four compounds. Then, the established method was applied to detect the concentration of these four compounds in SHT samples from two

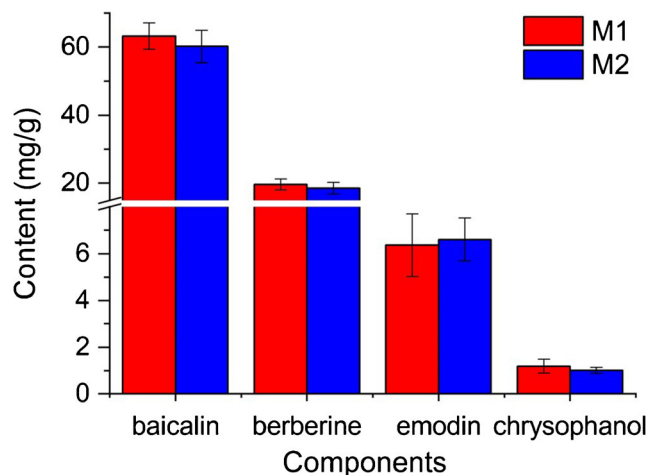


Fig. 5 The contents of the four components in SHT samples from two different manufactories

manufactories. As shown in Fig. 5, no significant differences in the contents of these compounds were observed between the test samples.

Pseudotargeted analysis of SHT samples using the established method

Fig. S3B and C (see ESM) show that no obvious differences between SHT samples from two manufactories could be observed from their chromatograms. And Fig. 5 exhibits no significant diversities in the contents of these four compounds between the SHT sample from two manufactories, suggesting there is no quality difference in test samples.

SHT samples from two manufactories were analyzed by the developed pseudotargeted method. The integrated areas of 85 peaks were used for the next statistical analysis. PLS-DA model was conducted with R^2Y and Q^2 values of 0.996 and 0.986, respectively, to discriminate the two groups according to differences in their component compositions and screen out the potential analytical markers for the SHT quality assessment. As shown in Fig. 6a, the clear classification between the two groups observed in PLS-DA score plot reflected the chemome differences of the SHT samples from two manufactories, suggesting the quality variety among test

Table 1 Statistical results of the validation of four compounds

Compounds	Precursor ion (m/z)	Quantitative ion (m/z)	Linearity			LOD (ng/mL)	Precision (RSD, %, $n=6$)
			Range (ng/mL)	Regression equation	r		
Baicalin	447.08	271.06	13.5–13,500	$y = 4.1086 \times 10^6 x + 1.4794 \times 10^7$	0.9951	0.68	3.41
Berberine	336.12	321.10	4.2–2100	$y = 2.9162 \times 10^7 x + 1.4804 \times 10^8$	0.9994	0.14	4.65
Emodin	271.06	197.06	9.4–1880	$y = 1.7682 \times 10^6 x + 2.6112 \times 10^7$	0.9977	0.47	3.16
Chrysophanol	255.06	227.07	2.1–1050	$y = 4.7973 \times 10^6 x + 2.2509 \times 10^7$	0.9991	0.70	2.42

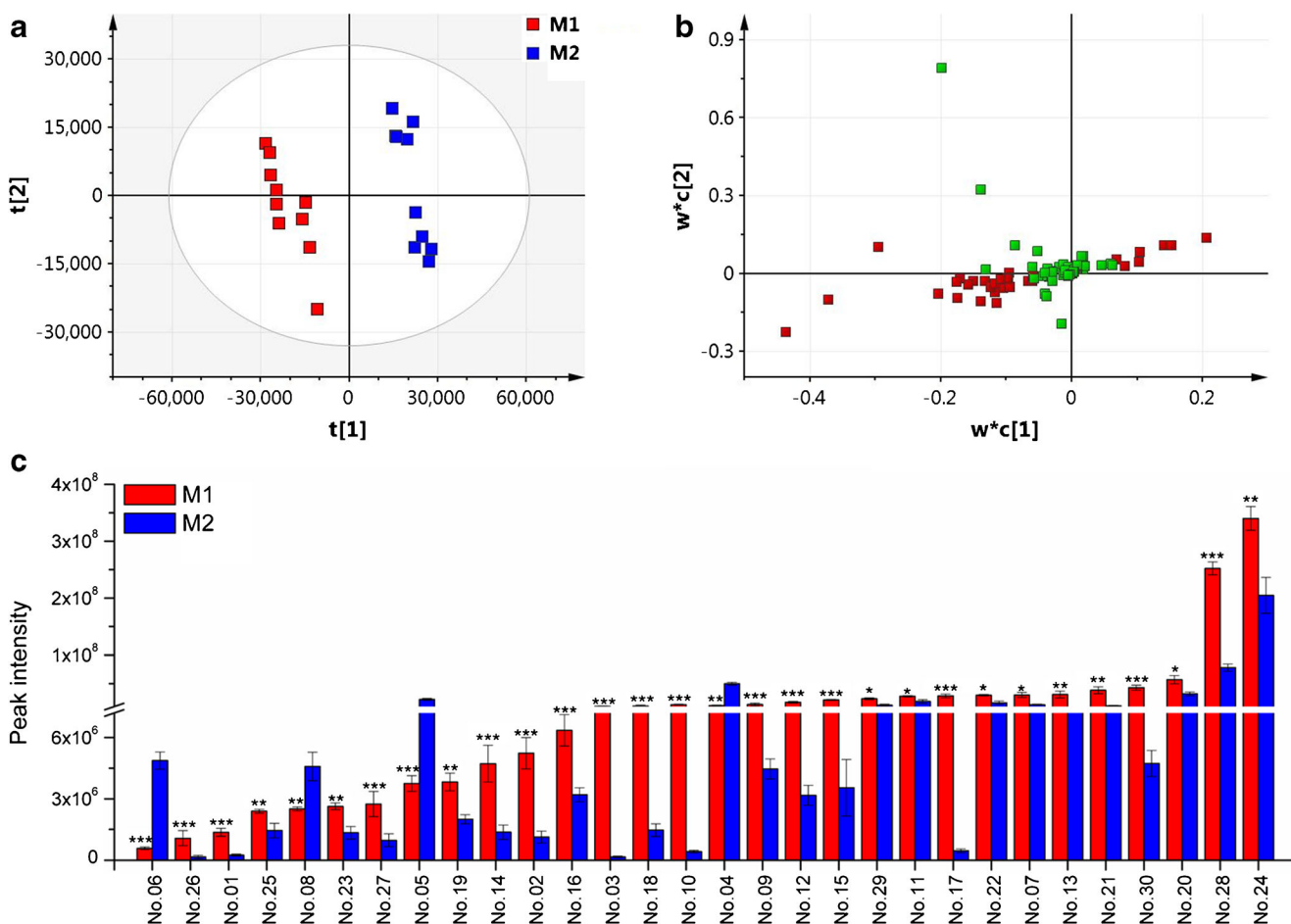


Fig. 6 PLS-DA score plot (a), and the corresponding loading plot (b) of SHT samples. Potential analytical markers (VIP1 > 1, VIP2 > 1, $p < 0.05$) were marked with red color in the loading plot, and their intensity changes are shown in c (*, **, and *** $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively)

samples, which is not consistent with the aforementioned quantification analysis.

To explore the chemical components that contribute most to the separation of SHT samples from different manufactories, the loading plot and variable importance in the projection (VIP) were constructed and generated from PLS-DA. Each point on the loading plot in Fig. 6b represents the one component detected in SHT samples, and the further components are away from the origin, the stronger they contribute to the discrimination. The features with VIP1 and VIP2 large than 1 were selected initially as important markers for discrimination, and these ions were further screened by Student's t test to select the components with a significant difference between two groups. Finally, thirty ions with $p < 0.05$ were kept as the analytical markers and marked with red color in the loading plot. The differences in the intensity of these analytical markers between SHT samples are shown in Fig. 6c.

The identification of analytical markers was performed by matching the accurate m/z and MS/MS information obtained from standard reference or recorded in literature [19–22]. Here

the tentatively annotated as aloe-emodin-8-O-beta-D-glucopyranoside ion at RT 4.71_ m/z 455.09 was used as an example to demonstrate the identification procedures for the compound without standard reference. As shown in Fig. 7a, the ion at m/z 455.09 corresponding to the $[M+Na]^+$ ion of aloe-emodin-8-O-beta-D-glucopyranoside. The most intensive product ion at m/z 293.04 results from the loss of $C_6H_{10}O_5$. And other fragment ions at m/z 185.04, 247.04, and 275.03, corresponding to the loss of $C_{15}H_{10}O_5$, $C_7H_{12}O_7$, and $C_6H_{12}O_6$, respectively. The possible fragmentation pathways for the product ions are shown in Fig. 7b. Finally, nine analytical markers were tentatively annotated by examining their MS spectra and MS/MS fragmentation patterns and are summarized in Table 2. And these results were consistent with the previous studies.

Discussion

Baicalin, berberine, emodin, and chrysophanol are the main active components in SHT and have been used to conduct

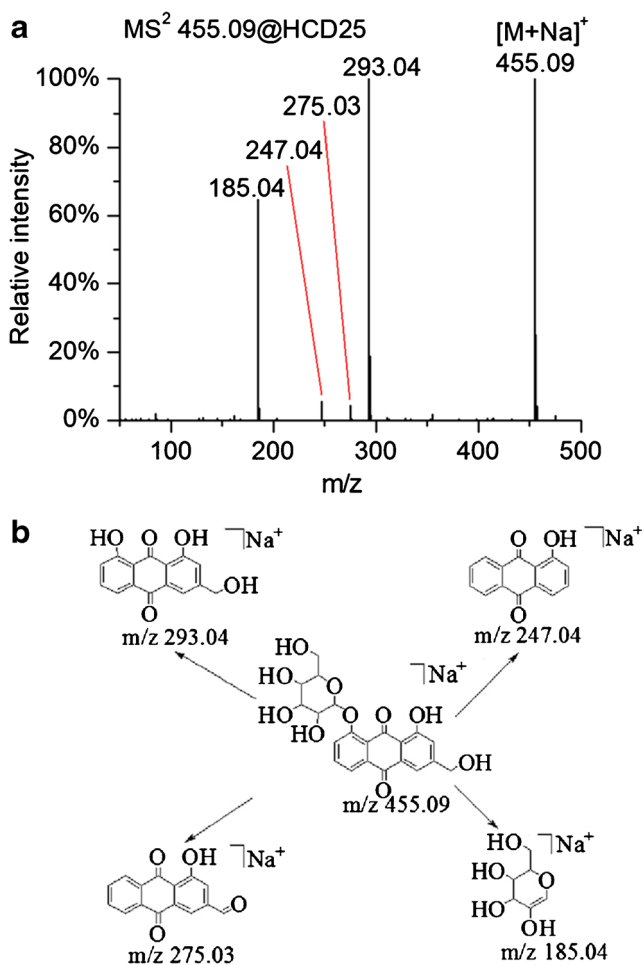


Fig. 7 The MS/MS spectrum (a) and possible fragmentation pathways of sodium-adducted aloe-emodin-8-O-beta-D-glucopyranoside ion (b)

qualification and quantification for SHT. No significant differences in the contents of these four compounds are shown in Fig. 5, which indicates that there is no quality diversity presented in test SHT samples from two manufactories. However, all samples were separated into two groups by multivariate statistical analysis, and 30 analytical markers that contributed most to the discrimination were screened out and their intensity changes in different groups are shown in Fig. 6c. Among these markers, 4 compounds (Nos. 4, 5, 6, and 8) in the M2 group showed higher intensity than those in the M1 group, but the relative low intensity was observed in the M2 group for other 26 compounds compared with that in the M1 group. The results indicated that the quality control for the SHT is not sufficient just by analyzing four main active compounds, and several components highlighted from pseudotargeted analysis could offer a complementary factor for the TCHP quality assessment.

Pseudotargeted analysis integrates the merits of both untargeted and targeted analyses and has been applied in many research fields [23–25]. Conventionally, an HRMS for MS/MS information collection and a QQQ MS for ion pair monitoring

are needed to perform the pseudotargeted analysis. Considering the time consumption in the chromatogram separation method transformation and ion pair pickup procedures, we used an LC-Q-Orbitrap/MS operated in PRM mode to achieve the pseudotargeted analysis. Compared with the previous tsMIM-based pseudotargeted method, which can also be completed by one MS instrument, the shortage of the loss of MS/MS information for component identification was covered by the introduction of PRM analysis [16]. And the tsPRM was applied to ensure the adequate scan point of each target ion, and further improve the accuracy of quantitative analysis especially for the ion with low intensity.

The data-independent acquisition (DIA) techniques, including (sequential window acquisition of all theoretical fragment ion spectra) SWATH and MS^E, were also applied to obtain MS and MS/MS information of analyte in one analytical run, and further to complete the qualification and quantification [26, 27]. One of the differences between DIA and DDA methods is the precursor selectivity. Our established PRM-based pseudotargeted method belongs to the DDA, which has the greatest precursor selectivity, while the MS^E possesses the worst due to the widest m/z window applied during the analysis. The SWATH approach usually uses m/z 25 consecutive isolation window to cover the whole acquisition mass range, which improves the precursor selectivity. However, both MS^E and SWATH will not create direct link between a specific precursor and its product ions, when analyzing complex samples, in which the analyte with similar structures and retention time cannot be separated completely, and often be isolated and triggered in the same MS/MS scan [28–30]. The established PRM-based method covers a wide mass range with better precursor selectivity, which could improve quantitative performance in the analysis of complex samples by reducing the fragment interference. But for the analysis of less complex samples such as multiple step extracted plant sample, our method may not exhibit superior over existing approaches. Of note, since no time-staggered ion lists were needed for less complex sample detection by the established method, the time-consuming will be reduced, which makes it more applicable in sample analysis.

Besides, the developed tsPRM method is less throughput due to the use of time-staggered ion for detection, which costs a threefold time higher than untargeted method. In addition, the relative slow scanning speed of orbitrap MS only permits monitoring of a limited number of targeted ions during a certain time interval. When a large number of ions monitoring is needed for example the biological sample with complex matrix background, we recommend dividing the original ion list into more number of sublists, or transferring the tsPRM-like pseudotargeted method from an orbitrap MS instrument to a Q-TOF MS instrument that possesses higher scan speed.

Table 2 The detailed information of analytical markers

No.	RT (min)	Measured m/z	Mass deviation (ppm)	Adducts	Product ions	Compounds	Elemental composition	References
1	4.71	455.0946	-0.4	[M+Na] ⁺	293.04, 185.04	Aloe-emodin-8-O-beta-D-glucopyranoside ^b	C ₂₁ H ₂₀ O ₁₀	[20]
2	4.71	271.0598	-1.1	[M+H] ⁺	225.06, 253.05, 241.05	Norwogonin ^b	C ₁₅ H ₁₀ O ₅	[21]
3	4.80	549.1599	-	-	393.10, 411.11, 375.09	-	-	-
4	4.87	131.0492	-	-	103.05	-	-	-
5	4.87	293.1015	-	-	131.05	-	-	-
6	4.87	333.0941	-	-	315.08	-	-	-
7	4.89	419.1333	-1	[M+H] ⁺	257.08	Barbaloin ^b	C ₂₁ H ₂₂ O ₉	[22]
8	5.05	352.1175	-	-	336.09, 308.09, 322.07	-	-	-
9	5.20	477.1022	-	-	301.07, 286.05	-	-	-
10	5.37	347.0757	-	-	332.05, 314.04, 169.01	-	-	-
11	5.53	433.1125	-	-	255.07	-	-	-
12	6.05	477.1021	-	-	286.05, 301.07	-	-	-
13	6.05	247.0961	-	-	229.09, 201.09, 214.06	-	-	-
14	6.05	409.1488	-	-	229.09, 205.09	-	-	-
15	6.13	285.0746	-2.2	[M+H] ⁺	270.05, 271.05, 241.05, 183.03	Physcion ^b	C ₁₆ H ₁₂ O ₅	[19]
16	6.19	439.0993	-1.4	[M+Na] ⁺	277.05, 185.04	Daidzin ^b	C ₂₁ H ₂₀ O ₉	[19]
17	6.20	287.0546	-	-	183.03	-	-	-
18	6.20	301.0702	-	-	286.05	-	-	-
19	6.76	469.1102	-	-	307.06, 185.04	-	-	-
20	6.76	285.0755	-1.1	[M+H] ⁺	211.07, 242.06, 270.05	Oroxylin A ^b	C ₁₆ H ₁₂ O ₅	[19]
21	6.81	301.0703	-	-	286.05, 202.01	-	-	-
22	6.85	287.0545	-	-	241.05, 269.04, 225.06	-	-	-
23	6.85	291.0258	-	-	263.03, 243.06	-	-	-
24	6.85	271.0596	-1.8	[M+H] ⁺	123.01, 103.05	Baicalin ^{a,b}	C ₁₅ H ₁₀ O ₅	[19]
25	6.85	327.0471	-	-	237.05, 265.05	-	-	-
26	7.34	473.1412	-	-	269.08, 227.05	-	-	-
27	7.55	481.1099	-	-	227.05, 277.05	-	-	-
28	8.03	285.0756	-0.7	[M+H] ⁺	270.05, 271.05	Wogonin ^{a,b}	C ₁₆ H ₁₂ O ₅	[19]
29	8.04	255.0651	-0.4	[M+H] ⁺	153.02	Chrysin ^b	C ₁₅ H ₁₀ O ₄	[22]
30	8.39	375.1073	-	-	345.06, 327.05	-	-	-

^a The identification was confirmed by standard. ^b This compound has been reported previously

Conclusions

In this study, we proposed a pseudotargeted analysis method using a UHPLC/Q-Orbitrap/MS operated in the PRM mode, which exhibits better repeatability, higher sensitivity, and wider linear range than the full scan analysis. Moreover, the introduction of tsPRM could greatly improve the peak shape and the accuracy of the compound quantification by increasing the number of scan points. By the successful application of this method in the discovery of analytical markers of SHT, the established method was proved to have great potential in quality assessment of TCMP.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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